Use of tissue chromogranin A as chronic and acute stress marker in fish

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ABSTRACT

Chromogranin A (CgA) has recently reported as stress marker in superior vertebrates. It is stored in granules of the chromaffin tissue and released to the bloodstream from the adrenal medulla and pituitary after stress situations. The objective of this work was to study the chromogranin A variation for acute and chronic stress in fish, aiming at determining if those proteins could be suitable stress markers. A chronic stress experiment was conducted consisting of two treatments, stressed and control meagres (*Argyrosomus regius*) for 6 months. The stressed groups were submitted to confinement and netting/chasing stress. The control group tanks were not disturbed along the experiment. A complementary acute stress challenge was performed exposing control fish to air for 3 min. Fish were sampled for blood, tissues and biometry. Plasma lactate and cortisol increased significantly after acute stress although glucose and proteins remained stable, and kidney cortisol and brain adrenaline were significantly higher. Kidney CgA decreased significantly in the acute stressed fish though brain CgA did not change. Final weight and length, growth and condition index were significantly lower in chronically stressed fish, though survival rate was not different between treatments. Plasma markers did not change significantly though kidney cortisol increased in chronically stressed fish. Brain noradrenaline was lower in chronically stressed fish. Both brain and kidney CgA concentrations decreased in stressed (chronic and acute) fish. Concluding, only kidney CgA and cortisol kept the same variation pattern in both stress types. Although cortisol concentrations in plasma and tissues have been widely studied, the tissue CgA concentrations related to stress have not still reported in fish. Initially, the depletion of kidney CgA could be considered as a chronic stress marker though it needs to be supported by future research.

1. Introduction

The physiological variables which indicate teleost fish stress are very diverse; in fact, any parameter indicating deviation from the homeostasis status could be considered as a stress indicator. The most part of those markers are linked to the endocrine response. Overall, this initially consists of a response called General Adaptative Syndrome (GAS), a hormonal cascade which leads to other responses to stressors (Schreck and Tort, 2016). The HPI (Hypothalamus-Pituitary-Interrenal) and HSC (Hypothalamus-Sympathetic-Chromaffin) are activated for this primary response, releasing corticosteroids (mainly cortisol) and catecholamines (adrenaline, noradrenaline and dopamine) to the bloodstream. Then, the above-mentioned indicators are detectable in blood and tissues, as described by Salamanca et al. (2021). Following, several intermediary metabolism pathways are enhanced, which is the stress secondary response and, if stress persists (distress), severe fails at organism level

appear (Iwama et al., 2006; Schreck and Tort, 2016). The secondary responses are mainly assessed studying the circulation and transformation of reserve substances through blood/tissues and enzyme activity analyses (Sangiao-Alvarellos et al., 2005; Herrera et al., 2015). The tertiary responses are identified by general dysfunctions coming from multiorgan failures, such as growth decrease, mortality, lower resistance to pathogens, losses in the reproductive performance, etc. (Madaro et al., 2015; Refaey et al., 2018).

The meagre (*Argyrosomus regius*) is a relatively new species in aquaculture research, though its stress responses have been previously studied, being like other teleosts (Fanouraki et al., 2011; Samaras et al., 2016; Fernández-Alacid et al., 2019; Asencio-Alcudia et al., 2019; Monteiro et al., 2021; Salamanca et al., 2021; Herrera et al., 2020, 2021). However, some authors have stated that the differences between basal and stressed values of stress markers are lower than other fish species, though significant (Fanouraki et al., 2011; Samaras et al., 2016).

The primary stress markers based in the HPI reactivity (cortisol, corticotropin releasing hormone) are much more used than those deriving from the HSC. It is due to HSC indicators (mainly catecholamines) are released and removed quickly from the bloodstream after stress, meanwhile HPI markers are more stable (Barton, 2002). Nevertheless, it has been described that an HPI axis acclimation to chronic stress conditions can exist, hence the efficacy of those indicators is sometimes poor, despite there is no information on the physiological processes involved (Montero et al., 1999; Procarione et al., 1999; Haukenes and Barton, 2004; Barton et al., 2005). The circadian rhythms affecting cortisol release do not either support its efficacy as stress marker (Sánchez Vázquez et al., 2019). In this sense, the search of new stress markers based on the HSC axis response could be an interesting topic. As mentioned, the catecholamines reflect a prompt response and are quickly released and removed from the bloodstream, although there are some proteins released at the same time, the chromogranins, which are more stable in the blood (Srithunyarat et al., 2018).

Chromogranins belong to the granin family, a group of soluble acid proteins which express in several endocrine, neuroendocrine and neuronal cells (Taupenot et al., 2003a). Specifically, the biosynthesis of chromogranins has been described in cells from the gastrointestinal and respiratory tracts, adeno- and neuro-hypophysis, parathyroid glands, endocrine pancreas, and the adrenal medulla from humans, cows, mice, and rats (Taupenot et al., 2003a, 2003b; Helle et al., 2007; Bartolomucci et al., 2011; D'amico et al., 2014; Corti et al., 2018).

They are stored in secretory granules and released together with peptidic hormones, neurotransmitters or amines as response to a range of stimuli (D'amico et al., 2014). At intracellular level, chromogranins regulate the calcium and pH levels, also interacting with other biomolecules to form secretory granules (Taupenot et al., 2003a, 2003b). From an endocrinological perspective, it has been suggested that chromogranins act as prohormones, giving rise to several bioactive peptides. Hence, peptides derived from chromogranin A (CgA) would have autocrine, paracrine, and endocrine activities (Taupenot et al., 2003a, 2003b). For instance, pancreastin inhibits glucose-stimulated insulin and amylase releases, activates hepatic glycogenolysis, and inhibits vasoconstriction in superior vertebrates (Tatemoto et al., 1986; Aardal et al., 1993; Sanchez-Margalet et al., 2000; Cadman et al., 2002).

Moreover, CgA has recently reported as stress marker in tetrapods, and also humans (Toda et al., 2007; Escribano et al., 2013; Srithunyarat et al., 2016, 2018). This glycoprotein is stored in granules of the chromaffin tissue and released into the bloodstream from the adrenal medulla and pituitary after stress situations (Taupenot et al., 2003a, 2003b). In this sense, CgA and/or derived peptides can modulate the release of catecholamines from sympathoadrenal chromaffin cells and inhibit the secretion of proopiomelanocortin hormone (Wand et al., 1991; Mahata et al., 1997; Taupenot et al., 2000). Blood CgA concentration is more stable than catecholamines, not being submitted to circadian rhythms. These facts, together with its easier analysis and preservation, make them a remarkable candidate for stress markers based on the SAM (sympathetic-adreno-medullar) axis reactivity, as described in pigs and other animals (Hayashi et al., 2014; Martínez-Miró et al., 2016).

Although the features of peptides derived from chromogranins acting as cardiac stabilizers have been studied in fish, their relationship with the stress response has been not described to the best of our knowledge (Imbrogno et al., 2019). In this sense, it has been reported that these peptides show cardio-inhibitors effects in teleosts and use several strategies for the cardiac control, despite its biological meaning still remains unclear (Mazza et al., 2007; Imbrogno et al., 2017).

From the first CgA sequence, coming from bovines, the expression, structure and function of chromogranins (CgA and CgB) have been studied in several species. Therefore, their presence have been demonstrated in the whole animal kingdom including invertebrates, highlighting their high phylogenic preservation (Bartolomucci et al., 2011). Despite existing differences in the amino acid composition among species, some specific parts of those molecules have a high level of amino acid homology, hence the analytical methods which antibodies direct to specific epitopes can use to measure samples of different species (Stridsberg and Angeletti, 2000).

Therefore, the objective of this work was to study the chromogranin A variation for stress in meagre, aiming at determining if those proteins could be a suitable stress marker, mainly for chronic stress conditions.

2. Material and methods

2.1. Experimental cultures and sampling

The experimental cultures were carried out at the IFAPA Agua del Pino (Cartaya, Spain) facilities. 900 L circular tanks (0.65 m radius) integrated in a close water system were used. Culture water was depurated with sand, physical, UV and biological filters. The water temperature and dissolved oxygen were checked daily, the temperature being constant at 20 °C (20 ± 0.06 °C, mean ± SEM) through a heat exchanger, and the dissolved oxygen above 5 ppm (6.57 ± 0.03 ppm; 88.8 ± 0.5% air saturation) by means of air stones. Photoperiod was natural, with a light intensity of 500–1000 lx. The renewal rate was 400% daily. The feeding consisted of commercial fish feed (L3 Alterna® Skretting, Burgos, Spain: 46.5% protein, 20% fat, 8.4% ash, 4.3% cellulose, 1.4% total phosphorous, 18 MJ Kg^{-1} energy) *ad libitum*, being 2–3% tank biomass.

Meagre juveniles were purchase from MARESA (Ayamonte, Spain) and submitted to an acclimation period in the above conditions for two weeks (20 fish tank^{-1}). After, fish were initially sampled for body weight (181.5 ± 15.1 g) and total length (37.3 ± 0.88 cm). There were two experimental procedures: chronic stress culture and acute stress challenge.

Chronic stress culture: It consisted of two treatments (per duplicate), stressed (CS) and control (CT) for 6 months. The CS groups were submitted to confinement and netting/chasing stress. Those tanks had a water column of 20 cm, thus an initial stocking density of 14 Kg m^{-3}. Additionally, fish were randomly net-chased and netted (with no exposure to air) daily for 5 min twice a day. The CT group tanks had a water volume of 500 L (40 cm water height, 7 Kg m^{-3}) and were not disturbed along the experiment. All tanks were supervised daily for cleaning and possible dead fish.

Acute stress challenge: After completing the chronic stress culture, all remaining fish from the control groups were kept in the same non-stressing culture conditions. After 15 days, 10 fish were netted and exposed to air stress for 3 min, taken back to tanks and sampled after 15 min.

Fish were sampled at the end of the experimental chronic stress culture (6 months), and after the air stress challenge (see above) for blood, tissues and biometry. Fish were sacrificed with 2-phenoxyethanol (1 mL L^{-1}). Blood was collected by puncturing the caudal peduncle with 1 mL heparinized syringes (25,000 units of ammonium heparin 3 mL^{-1} of 0.6% NaCl saline, Sigma H6279). Plasma was separated from cells by centrifugation of whole blood (10 min; 1200 ×g; 4 °C), and stored at −80 °C until the analysis of glucose, lactate, protein, and cortisol and catecholamine hormones was performed. The brain and a portion of head kidney were removed from each fish and stored at −80 °C until analyzed. Besides mean final weight and length, several zootechnical parameters were calculated: Specific Growth Rate, SGR = 100· (ln Wf – ln Wo)/t; condition factor, K = BW / TL^3, and survival rate (Wf, Wo, t, BW and TL are final and initial weight, time, final body weight and total length, respectively).

The IFAPA facilities are certified and have the necessary authorization for the breeding and husbandry of animals for scientific purposes (REGA code ES210210000303). All procedures involving the handling and treatment of the fish were approved as far as the care and use of experimental animals are concerned, by the European Union (2010/63/EU) and the Spanish Government (Royal Decree 53/2013, February 1,

on the basic guidelines for the protection of animals used for experimentation and other scientific purposes, including training).

2.2. Plasma analysis

Plasma glucose, proteins, lactate, adrenaline and noradrenaline levels were measured using commercial kits from Química Analítica Aplicada S.A. (QCA Glucose Liquid Ref. 998,225, QCA Total Proteins Ref. 997,180, Tarragona, Spain), Spinreact (Lactate Ref. 1,001,330, Barcelona, Spain), and 2-CAT (A-N) Research ELISA (Ref. BA E-5400, Nordhorn, Germany) adapted to 96-well microplates (Herrera et al., 2012; Salamanca et al., 2021). Plasma chromogranin A concentration was determined through an ELISA commercial kit (FineTest ChgA ELISA Kit, ref. ER0468, Wuhan Fine Biotech, Wuhan, China), 31.25 pg mL^{-1} being the lower limit. The inter-assay coefficient of variation was 11.1%, while the mean intra-assay coefficient of variation was 9.7%. No significant cross-reactivity or interference has been observed for this ELISA method.

All assays were performed with a Tecan Sunrise microplate reader, using Magellan v2.5 software for Windows (Tecan Austria, Salzburg). Plasma cortisol levels were quantified by an ELISA kit (EA65, Oxford Biomedical Research, MI, USA) modified and adapted to fish (Herrera et al., 2014). Cortisol was extracted from 20 μL plasma in 200 μL diethyl ether. The lower limit of detection (88.2% of binding) was 0.005 ng mL^{-1} plasma. The inter-assay coefficient of variation was 9.8%, while the mean intra-assay coefficient of variation was 4.6%. The mean percentage of recovery was 90%. The main cross-reactivities (>5%; given by the supplier) were detected with prednisolone (66.9%), 11-deoxycortisol (58.1%), cortisone (15.9%), prednisone (13.7%), and 17-hydroxyprogesterone (5.4%).

2.3. Tissue analysis

For brain catecholamines and chromogranin A concentrations, 20–50 mg of brain tissue was homogenized by ultrasonic disruption with 150 μL of perchloric acid (PCA), and then mixed with 150 μL of potassium dichromate 0.15 M. Following this, the mixture was centrifuged (1200 g; 4 °C; 10 min) and the supernatant removed. The precipitate was air dried for 2 h at 25 °C. Finally, 250 μL of distilled water was added to the tube and the solution used for ELISA kit determination. Adrenaline, noradrenaline and dopamine were measured through the 3-CAT Research ELISA (Ref. BA E-5600, LDN, Nordhorn, Germany, sensitivity of 0.25 ng mL^{-1} for adrenaline and dopamine, and 0.1 ng mL^1 for noradrenaline), as reported in Salamanca et al. (2021). And chromogranin A concentration was determined by means of the FineTest ChgA ELISA Kit (Ref. ER0468, Wuhan Fine Biotech, Wuhan, China, sensitivity of 31.25 ng mL^{-1}). The inter- and intra-assay coefficients of variation were lower than 10 and 8%, respectively. Head kidney was also homogenized and analyzed for cortisol and chromogranin A concentrations according to the above procedures.

2.4. Statistical analysis

Normality and homoscedasticity of all data sets were checked through the Kolmogorov–Smirnov and Levene tests, respectively. Differences among treatments were detected through a Student's *t*-test (normal variables) or U–Mann Whitney (non-normal variables) test. Data are expressed as mean ± standard error of mean ($n = 7$–10). The significance level was 0.05.

3. Results

3.1. Acute stress

Within the classical stress markers, lactate and cortisol increased significantly 15 min post-stress although glucose and proteins remained

stable (Fig. 1 and Fig. 2). Plasma lactate after acute stress grew threefold over non-stressed values (7.19 ± 1.24 *versus* 21.1 ± 1.73 mg dL^{-1}). Similarly, plasma cortisol in stressed fish was 84.1 ± 8.49 ng mL^{-1}, significantly higher than 32.5 ± 9.26 ng mL^{-1} for non-stressed fish. As plasma cortisol, kidney cortisol after stress was significantly higher, 0.37 ± 0.01 *versus* 0.23 ± 0.04 pg g^{-1} (Fig. 2). Regards catecholamine concentrations in brain, only adrenaline increased significantly after stress, being 30.7 ± 4.11 and 16.4 ± 3.13 pg g^{-1} for stressed and non-stressed fish, respectively (Fig. 3).

Plasma chromogranin A (CgA) concentrations were not detected through the ELISA kit (values under the detection limit). Kidney CgA in stressed fish was significantly lower (0.64 ± 0.04 pg mg^{-1}) than in non-stressed fish (10.3 ± 2.91 pg mg^{-1}); however, brain CgA did not change significantly between treatments (Fig. 4).

3.2. Chronic stress

Several biometric parameters varied between treatments significantly (Table 1). Final weight and length, growth and condition index were significantly lower in chronically stressed fish, though survival rate was not different between treatments (75 ± 5 and 68 ± 2.5% for non-stressed and stressed groups, respectively). Indeed, growth rate in control fish was approximately twice the chronically stressed one (0.41 ± 0.04 and 0.22 ± 0.08 day^{-1}, respectively).

Plasma stress markers did not change significantly (Fig. 1 and Fig. 2). However, kidney cortisol increased in stressed fish (0.42 ± 0.01 *versus* 0.23 ± 0.04 pg g^{-1}). Brain catecholamine concentrations only changed for noradrenaline (Fig. 3). This hormone concentration dropped from 98.3 ± 7.91 in control fish to 42.8 ± 6.85 pg g^{-1} in chronically stressed fish. Spite of adrenaline increased in stress status; this was not significant.

Both brain and kidney CgA concentrations decreased in stressed fish (Fig. 4). However only the kidney CgA variation was significant (from 10.3 ± 2.91 to 1.22 ± 0.3 pg g^{-1}).

4. Discussion

In this work, the determination of CgA concentrations in meagre (*Argyrosomus regius*) tissues has been reported for the first time. Only two previous works have studied the concentrations of chromogranin-derived peptides in fish hearts (through *in vivo* preparations), stating their role as cardiac modulators (Mazza et al., 2007; Imbrogno et al., 2017).

As a result, the efficacy of CgA as stress marker in fish has been assessed for the first time. Both acute and chronic stress responses led to a tissue CgA decrease, especially in kidney. Plasma CgA values were not detected due to the sensitivity of the ELISA method, hence they would be lower than 31.25 pg mL^{-1} (the kit lower limit). To our knowledge, no work has stated that circulating chromogranins in plasma are bound to proteins (Taupenot et al., 2003a, 2003b; Helle et al., 2007; Bartolomucci et al., 2011; D'amico et al., 2014; Yang et al., 2015; Corti et al., 2018; Malczewska et al., 2020). Therefore, the method detections (mainly ELISA) are usually based on the use of antibodies detecting various epitopes of the plasma free-protein surface (Malczewska et al., 2020). It seems that, in our case, a more sensitive ELISA should have been used.

4.1. Acute stress assay

The acute stress responses are usually measured by plasma analysis, mainly lactate, glucose and cortisol (Vargas-Chacoff et al., 2011; Herrera et al., 2012; De la Roca et al., 2017). Other works have also assessed the changes in plasma catecholamines since reflect the HSC axis reactivity (Salamanca et al., 2021; Datta et al., 2022). Our results have shown that the experimental conditions caused acute stress responses in the fish since several plasma indicators increased significantly 15 min after the stress challenge (Table 2). Additionally, kidney cortisol also increased its
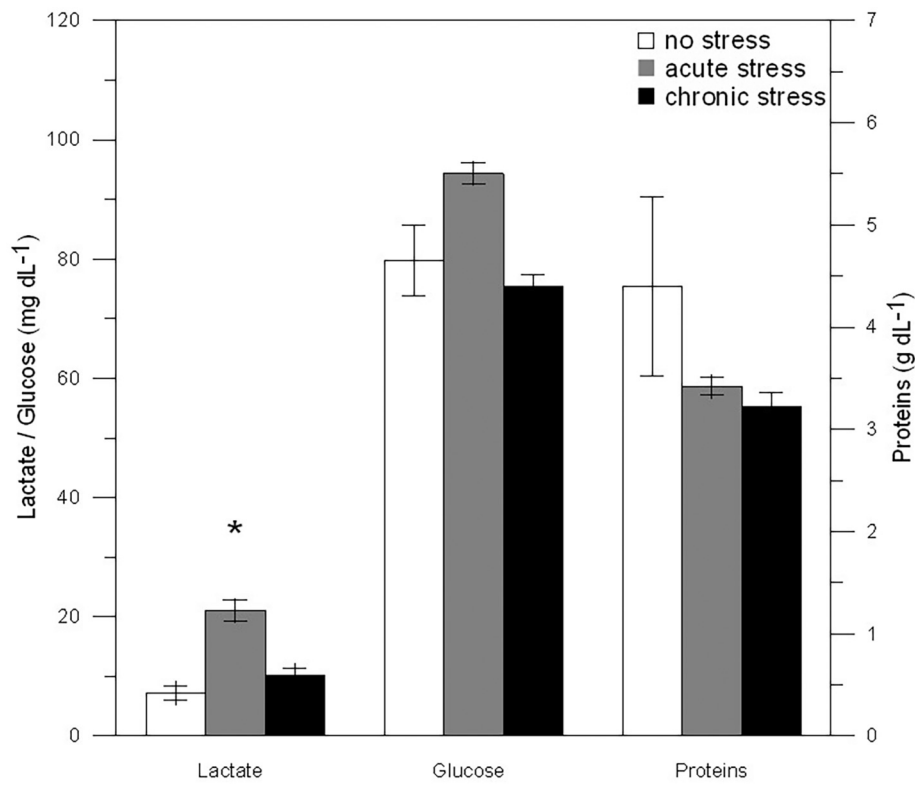
Fig. 1. Plasma glucose, lactate and protein concentrations measured after stress treatments. Asterisk (*) and number sign (#) indicate significant differences from the control (no stress) for acute and chronic stress, respectively ($p < 0.05$).

Fig. 2. Plasma and kidney cortisol concentrations measured after stress treatments. Asterisk (*) and number sign (#) indicate significant differences from the control (no stress) for acute and chronic stress, respectively ($p < 0.05$).

Fig. 3. Brain adrenaline, noradrenaline and dopamine concentrations measured after stress treatments. Asterisk (*) and number sign (#) indicate significant differences from the control (no stress) for acute and chronic stress, respectively ($p < 0.05$).

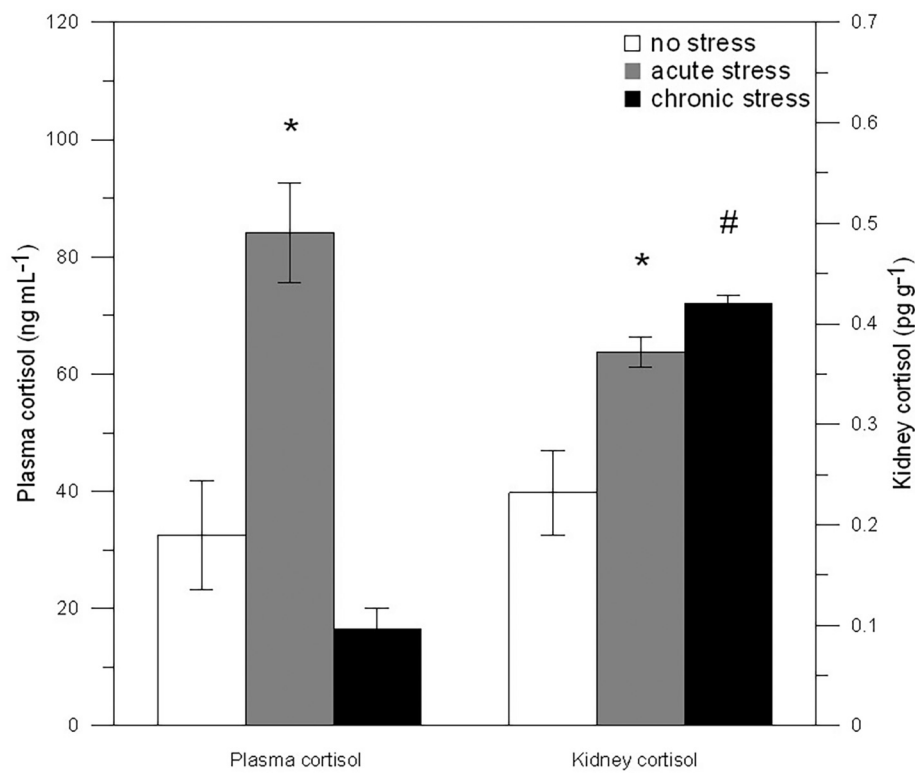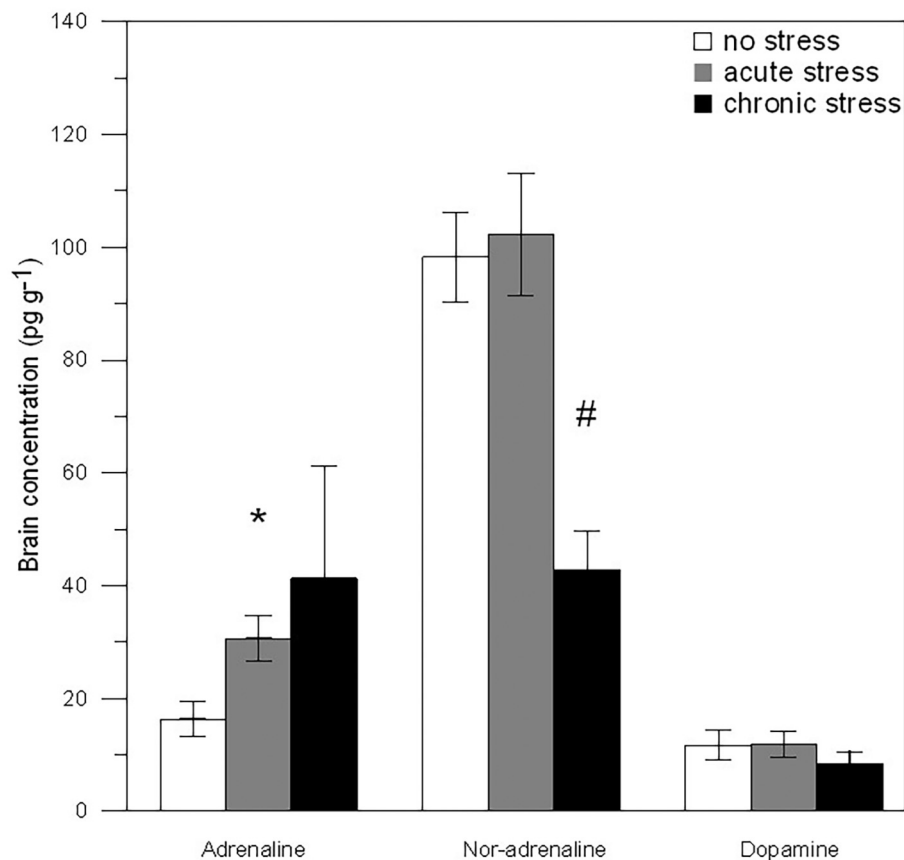concentration significantly along with the other markers. To the date, few works have studied the changes in kidney cortisol concentration, spite of being produced and released from that tissue (Mommsen et al., 1999). However, several works have focused on the gene expression of cortisol receptors and precursors from kidney samples (Herrera et al., 2021; Samaras and Pavlidis, 2018), agreeing that stressors enhance them. Therefore, our stress conditions derived in a quick stress response, at both plasma and tissue level.

The catecholamines are released and cleared quickly in the bloodstream during stress conditions, hence sometimes these are not reliable stress indicators (Barton, 2002). However, recent studies have demonstrated that they can be useful linked to other indicators if a suitable sampling methodology is followed (Salamanca et al., 2021, 2022). In our work, plasma adrenaline grew significantly after stress, confirming the stress status, as the cortisol variations pointed out previously.

In superior animals, CgA is present in the chromaffin granules of endocrine cells, neurons, and neuroendocrine cells (Di Comite and Morganti, 2011). According to the present work, CgA can also be detected in head kidney and brain, hence those proteins could come from the fish chromaffin cells and neurons, as said above. Tissue CgA concentration decreased after acute stress, although that change was only significant in the kidney. To the date, CgA have not been measured in animal tissues for assessing stress responses.

Srithunyarat et al. (2016) found a direct relationship between plasma CgA, cortisol and catecholamines in dogs after acute stress. In addition, salivary CgA in pigs submitted to restraint stress increased significantly (Escribano et al., 2015). This pattern has also registered in samples of human saliva and plasma (Nakane et al., 1998). In our work, the measurement of plasma CgA concentration was not possible due to the methodology applied although there were important decreases in tissue. To our knowledge, it has not been stated if CgA produced in

kidneys is released into the bloodstream or catabolized *in situ*. However, it has been reported that kidneys have a crucial role in eliminating CgA, since renal failures lead to a plasma CgA increase in humans (Hsiao et al., 1990; Takiyyuddin et al., 1990; Taupenot et al., 2003a, 2003b). Whereas fish kidneys also could influence CgA circulation significantly, the decrease in kidney CgA after stress in our work could be due to a relevant release of CgA into the bloodstream, supposing a quick depletion of CgA synthetized and/or stored at the kidney.

The pattern in brain was very similar, thus the distribution of these proteins to tissues through the bloodstream seems to be crucial during the stress response. It could explain that other biological samples as saliva and urine could also present high CgA concentrations after acute stress (Ott et al., 2014; Escribano et al., 2014, 2015; Huang et al., 2017).

4.2. Chronic stress assay

Fish submitted to chronic stress showed a significant lower growth. The loss of weight or slower growth are usually stress tertiary responses (Iwama et al., 2006), hence it is possible to confirm that confined fish were chronically stressed. It has been widely reported that chronic stress can alter growth rates (Pérez-Sánchez et al., 2018; Triantaphyllopoulos et al., 2020; Li et al., 2021). As mentioned above, it is a general response in many species to several stressor types. In that sense, chronic stress due to high stocking density led to lower growth rates in *Chelon labrosus*, *Scophthalmus maximus*, *Clarias gariepinus*, *Ictalurus punctatus* and *Oreochromis niloticus* (De Heras et al., 2015; Jia et al., 2016; Wang et al., 2017, Refaey et al., 2018; Zaki et al., 2020). Additionally, chronic stress due to thermal conditions or other different stressors also had negative effects on growth in *Labeo rohita* and *Salmo salar* (Kumar et al., 2014; Madaro et al., 2015). Nevertheless, sometimes the tertiary stress response has not been detected, with no changes in fish growth (Montero
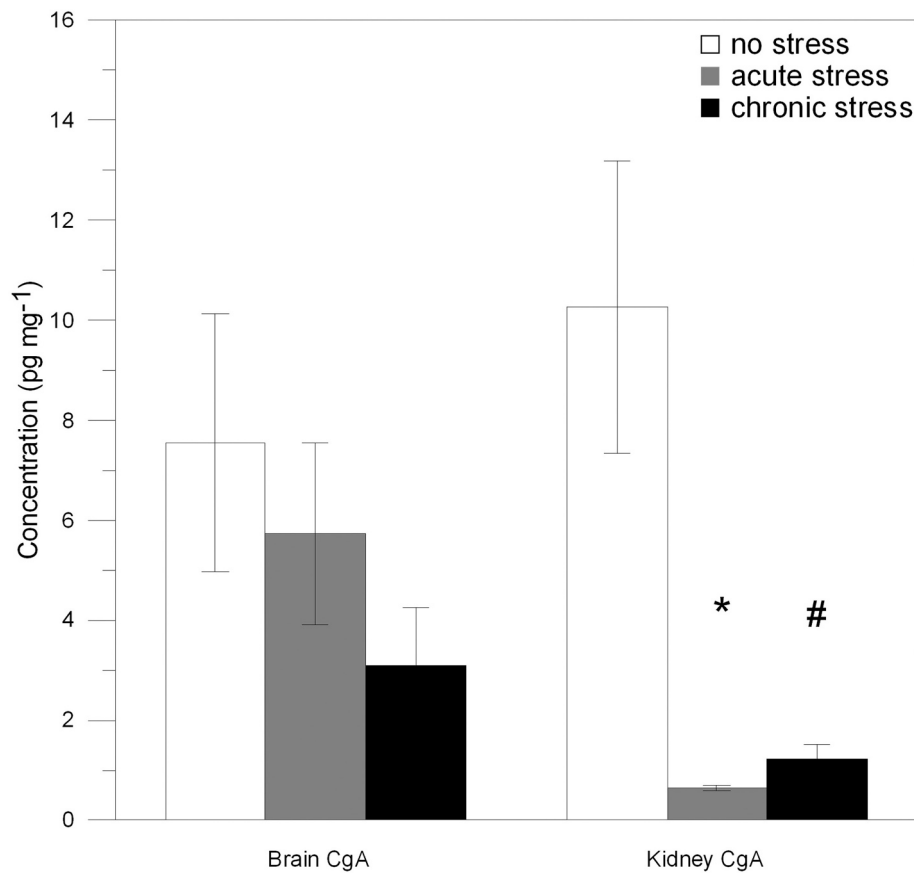
Fig. 4. Brain and kidney CgA concentrations measured after stress treatments. Asterisk (*) and number sign (#) indicate significant differences from the control (no stress) for acute and chronic stress, respectively ($p < 0.05$).

Table 1
Biometric parameters registered for non-stress and chronic stress treatments. Asterisks indicate significant differences between non-stressed and stressed status ($n = 10$; Student t-test; values are mean $\pm$ SEM).

	Non-stressed	Chronic stress
Initial weight (cm)	181.5	±15.1
Initial length (cm)	27.2	±0.88
Final weight (g)	403.1 ± 29.1 *	302.3 ± 46.8
Final length (g)	34.5 ± 0.93 *	31.5 ± 1.36
Specific Growth Rate (day^{-1})	0.41 ± 0.04 *	0.22 ± 0.08
K	0.94 ± 0.02 *	0.90 ± 0.02
Survival rate (%)	75 ± 5	68 ± 2.5

Table 2
Significant changes between control and stressed fish during the experiments. C = no significant change; R↑/ R↓ = significant increase/decrease regards the control (non-stressed) group.

Parameter	Stress type	
	Acute	Chronic
Biometric		
Growth	–	R↓
Final weight/length	–	R↓
K	–	R↓
Physiological		
Plasma glucose	C	C
Plasma lactate	R↑	C
Plasma proteins	C	C
Plasma cortisol	R↑	C
Kidney cortisol	R↑	R↑
Brain adrenaline	R↑	C
Brain noradrenaline	C	R↓
Brain dopamine	C	C
Brain CgA	C	C
Kidney CgA	R↓	R↓

et al., 1999a, 1999b; Costas et al., 2012; Herrera et al., 2015).

Contrarily to biometric variables, other classical stress markers as plasma cortisol, glucose and lactate did not change significantly in our experiment (Table 2). This is due to the HPI axis sometimes habituates to stress conditions and does not react through the release of metabolites and hormones into the bloodstream (Haukenes and Barton, 2004; Barton et al., 2005). This adaptation could be related to changes in corticosteroid receptors along the body during chronic stress conditions (Valenzuela et al., 2018; Jerez-Cepa et al., 2019). Indeed, that is one of the reasons why the search of new chronic stress markers is a subject of interest in the study of fish stress responses.

Kidney cortisol increased significantly in stressed fish, indicating that there was a high cortisol biosynthesis. No previous work on the relationship between kidney and plasma cortisol concentrations has been published. In fact, as indicated above, kidney cortisol analyses in the literature have been based on the expression of stress-related genes, reporting an over-expression of the most of them after stress submission

(Samaras et al., 2018; Herrera et al., 2021; Earhart et al., 2022). However, it seems that here the HPI axis did not react, as commented above (habituation), and hence some stress markers like cortisol were not released into the bloodstream, as the plasma results show. Therefore, probably kidney cortisol would be a more reliable stress indicator than plasma cortisol under chronic conditions.

Regards the CgA concentration, the pattern was similar to the stress acute experiment, both CgA brain and kidney decreasing. Previous works on superior animals have demonstrated the use of CgA

concentration as acute stress indicator (Toda et al., 2007; Escribano et al., 2013; Srithunyarat et al., 2016, 2018). Although its efficacy for chronic stress has not studied in animals yet, it has been reported that CgA and other chromogranins are valuable markers of psychological and psychosocial stress in humans (Kanamaru et al., 2006; Miyakawa et al., 2006; Tammayan et al., 2021; Liu et al., 2022). In our work, it seems clear that both chronic and acute stress led to the depletion of CgA in kidney and brain. Therefore, CgA could be released into the bloodstream from the main source (kidney), transferred and accumulated to other different organs as gut, liver or heart (D'amico et al., 2014). In this sense, CgA and derived peptides have been reported as cardiac stabilizers in fish, which could help to cope with stress situations since these alter significantly several cardiac variables as heart rate and stroke volume (Brijs et al., 2019).

5. Conclusions

According to our experiments, only kidney CgA and cortisol kept the same variation pattern in both stress types. Although cortisol concentrations in plasma and tissues have been widely studied, tissue CgA concentrations related to stress have not still reported in fish. Initially, the depletion of kidney CgA could be considered as a chronic stress marker though it needs to be supported by future research. Accordingly, both the development of more sensitive methods for analyzing plasma CgA and the analysis of CgA concentrations in other tissues and biological matrixes would be key tasks. As stress is closely linked to animal welfare, those matrixes should be based on non-invasive sampling protocols, thus studies on CgA determination in fish samples as scales, skin mucus and feces would be interesting future research lines.

Credit author statement

Herrera, M.: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing – original draft; Writing – review & editing

Salamanca, N.: Investigation; Data curation; Methodology; Validation; Final revision.

Ferrer, F.J.: Data curation; Methodology; Validation.

De La Rosa, I.: Conceptualization; Final revision.

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.

Data availability

Data will be made available on request.

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