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ORIGINAL PAPER

Remarkable variability in stress responses among subtropical coastal marine teleosts

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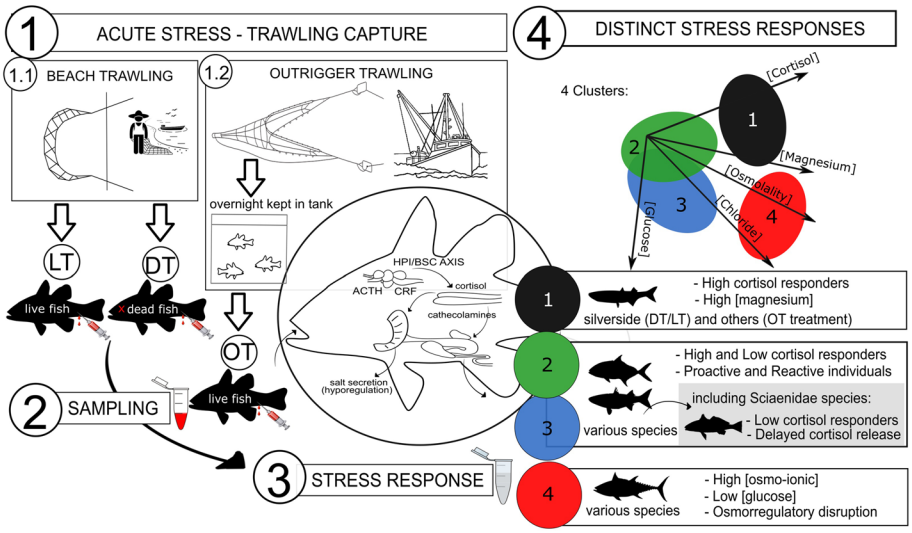
Abstract

The relevance of the plasma cortisol response as primary stress marker in teleost fishes is well known. However, available data still refer to a low number of species, considering the huge diversity of teleosts. To improve the data base of the cortisol response in feral coastal marine teleosts, this study aimed at evaluating the response—survival and plasma cortisol, glucose, osmolality, chloride, and magnesium—of marine teleosts to the capture stress of trawling. Fish sampled were divided into three groups: dead after trawling, alive after trawling, and live fish which were kept for ~ 12 h in a tank after trawling ("overnight in tank"). Blood samples (224) of 36 species from 19 fish families have been assessed. A large variability in the cortisol response among species, within the three groups, was detected. The Brazilian silverside *Atherinella brasiliensis* was notable in showing essentially only very high values (> 1000 ng/mL). Some species always showed very low levels (nearly zero, e.g., most Scianidae species). Most species displayed a very broad range of values (10–1000 ng/mL), such as *Eucinostomus argenteus*. Inter-specific variability was also present in plasma glucose, osmolality, and ions. The response to trawling stress in wild coastal marine teleost fish species is thus extremely variable. Better knowledge about the diversity in the response of teleosts to stress contributes to fisheries management, aiming at sustainability.

Graphic abstract



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Introduction

The stress response is an adaptive mechanism that allows fish to deal with real or perceived stressors, to assure the preservation of their homeostatic state, or even, under

intensely stressful conditions, their survival (Barton 2002; Schreck and Tort 2016; Sopinka et al. 2016; Winberg et al. 2016; Kalamarz-Kubiak 2018). In the short term, this response is considered adaptive because it initiates a set of physiological and behavioral reactions that promote the survival of the individual, through the activation and integration of mechanisms related to metabolism and homeostasis (Wingfield et al. 1998; Sapolsky et al. 2000). Hormones play key roles in the stress response of fish, especially the corticosteroids 11-deoxycortisol (in lampreys), 1a-hydroxycorticosterone (in elasmobranchs), and cortisol and other related steroids in chondrosteans and teleosts (Schreck and Tort 2016). The primary response to acute stress in teleosts in general comprises activation of the hypothalamic–pituitary–interrenal (HPI) axis and the fast release of catecholamines by chromaffin cells via sympathetic stimulation (Gorissen and Flik 2016; Schreck and Tort 2016; Winberg et al. 2016; Kalamarz-Kubiak 2018). Interrenal and chromaffin cells are located in the head kidney. Corticotropin-releasing factor (CRF) is released from the hypothalamus and stimulates the corticotrophic cells in the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH acts on corticosteroid-producing steroidogenic cells of the interrenal tissue, which then release cortisol to the bloodstream (Gorissen and Flik 2016; Schreck and Tort 2016; Winberg et al. 2016; Kalamarz-Kubiak 2018). This hormonal axis regulates daily metabolic needs as well as behavioral responses to environmental disturbances, in addition to regulating and integrating other routine functions of daily life (Winberg et al. 2016; Sopinka et al. 2016). In addition to the widespread recognition of the role of cortisol in the response to stress in fishes, other hormones are also reported to participate, such as arginine vasotocin, isotocin, and urotensins, as well as dopamine, serotonin, or beta-endorphin (Kalamarz-Kubiak 2018). In general, it may be considered that the intensity and/or frequency of activation of the stress response through the HPI axis may naturally influence the fitness of a species (Schoenle et al. 2018). Increase in plasma glucose by increased glycogenolysis, cardiovascular and respiratory responses, and osmoregulatory disturbances are generally considered secondary stress responses. These secondary responses mostly involve changes in metabolism, osmo-ionic homeostasis, immune-related and hematological factors (Iwama et al. 2006; Kalamarz-Kubiak 2018). Upon prolonged activation of the stress response, the so-called tertiary responses ensue, with reproductive and more intense immunological consequences, and thus, reduced fitness (Busch and Hayward 2009).

The evaluation of glucocorticoid levels raised great interest between the 1960s and 1970s (e.g., Bouck and Ball 1966; Donaldson and Dye 1975; Fryer 1975; Mazeaud et al. 1977). Years of study in the past 5–6 decades led to the production of a vast literature, recognizing an undisputed role of cortisol as a marker of stressful conditions for teleost fishes (e.g., see Mommsen et al. 1999). However, interpretations of circulating levels of glucocorticoids/cortisol in marine teleosts remain complex (Busch and Hayward 2009; Beaulieu and Constantini 2014; Kalamarz-Kubiak 2018). Recent wildlife investigations have revealed patterns of hormonal responses to environmental, physical, and social challenges, which could not have been predicted from laboratory experiments (Ajó et al. 2018; Currylow et al. 2018; Wingfield 2018). Simple 'linear' stress paradigms explored in laboratory contexts are unable to contemplate the inherent variability of the much more complex social (Goymann and Wingfield 2004), physiological, and physical interactions that occur in the natural environment (Pankhurst 2011).

New insights on the use of glucocorticoids as stress markers for wildlife have been recently proposed (Sopinka et al. 2015; Madliger et al. 2018), contrary to previous paradigms that the stress response could not really be contemplated in nature. These previous positions, contrary to the evaluation of stress responses in the natural environment, argued that the many influences from biotic and abiotic traits and the additional difficulty in obtaining "non-stressed" individuals would in fact prevent researchers from obtaining valuable data in the field. After much debate on how to apply an experimental "stressor" in the field, it was concluded that the vertebrate's response to capture, management, and containment could be a relatively standard response, which would approximately reflect the individual's ability to respond to an acute stressor (Wingfield 2018). It was then proposed that, if a blood sample is collected as soon as possible after capture (~ 3 min), then the plasma cortisol concentration in this sample at least approximately represents the baseline levels. This is a standard with a high degree of confidence for avian species, in which samples collected in less than 2 min reflect non-stressed concentrations (basal corticosterone) (Romero and Reed 2005). It is also considered a standard for teleost fishes, with blood glucose and plasma cortisol apparently remaining relatively low and stable within a 3-min sampling window (Bolasina 2011; Lawrence et al. 2018). Subsequent samples, after 5, 10, 30, and 60 min, for example, would properly illustrate the rates of increase and the maximum levels of cortisol obtained after acute stress (Wingfield 2018). Given that the way an animal responds to a short-term stressor may reflect its long-term ability to adapt to a changing environment in the face of diseases, anthropic disturbances, and increasingly scarce resources (Currylow et al. 2018), it is interesting and ecologically meaningful to improve our knowledge about the variability in the response to stress in wild fish.

Still, knowledge on the stress response of marine teleost fishes is confined to tens of species, which occur in countries with more developed fish ecophysiology research communities. Within these countries, there is a focus on species that are

economically or ecologically important and/or are relatively easy to obtain and maintain in captivity. These include species such as the Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Dover sole (*Solea solea*), European sea bass (*Dicentrarchus labrax*), Pacific salmonids of the genus *Oncorhynchus*, or the turbot (*Scopthalmus maximus*), plus various tropical species from the Great Barrier Reef (McKenzie et al. 2016). This current knowledge contrasts with the great diversity found in the fish group. Fish comprise the most diverse group of Craniata, with over 30,000 species (McKenzie et al. 2016). Therefore, in the literature, additional information on a wider array of species is of value (see Balasch and Tort 2019).

Anthropogenic pressures are shown to have global impacts on ecosystems, societies, and economies, threatening livelihoods, and food supplies, including those from fishing. Several collective human actions such as overfishing, overproduction of greenhouse gases, pollution, and habitat degradation contribute to significant decreases in commercially important fish populations (Sumaila et al. 2011; Cooke et al. 2013; Calosi et al. 2016; Britten et al. 2017; Madliger et al. 2017; Friedman et al. 2020).

Through this study, we have then proposed to investigate the degree of diversity in the acute stress response of marine coastal teleosts caught through coastal trawling, in the southern western Atlantic coast of Brazil. With a somewhat exploratory character, due to the paucity of information both on basal and induced levels of cortisol secretion of southern Atlantic species, this study will prompt further investigations regarding what is behind the inter-specific variability in HPI axis functioning. Knowing the resilience potential of fish in the face of these pressures is relevant to shape fisheries management plans, and species conservation. In this context, the goals of the study were as follows: (i) to evaluate the response of coastal marine fishes of several different families to capture stress, through the evaluation of cortisol, glucose, osmolality, and plasma ions; and (ii) to compare the level of the glucocorticoid response between fish that died from the trawling stress, fish that were recovered alive from the trawling net, and a third group, fish that were alive after trawling, and were kept overnight in a seawater tank. Osmolality and plasma ion measurements indicate important secondary physiological changes caused by stress due to their central role as mediators of homeostasis/allostasis, in addition to corroborating the post-stress plasma cortisol levels (Iwama et al. 2006; Takey and Loretz 2006).

Material and methods

Fish trawling and blood sampling

Fishes were obtained from the shallow platform coast of Paraná (25° 35′20.96″S and 48° 21′53.20″O) and Santa Catarina States (26° 18′38″S and 48° 52′76″W), in Southern Brazil, between April 2017 and July 2018. Two types of traditional artisanal fishing trawling were carried out—sandy beach hand trawling in the early morning (approximately 7 am) using a net 500 m-long, and 4 cm-mesh size (armed with the aid of a small boat and pulled by about 15–20 fishers), or outrigger trawling with a 10 m-long net of 3 cm-mesh size in the codend), during the evening (approximately 6 pm). Trawling duration was similar between both fishing gears (~ 30–45 min), with the longest times being related to the larger numbers of fish in the beach trawl, mainly during mullet catching seasons (June–July). Upon opening the net on the sand, fish were sorted out and divided into two groups: dead fish after trawling (DT) or live fish after trawling (LT). Blood samples were collected by caudal puncture using heparinized syringes, immediately after retrieving the fish from the net. Fish which were alive after trawling meant fish that were still showing opercular and caudal contractions when removed from the net. Only a few individuals were sampled from each trawl, so sampling was standardized as best as possible, avoiding that fish would be dead for over ~ 15 min. The third group, comprising a different treatment of the fishes (named OT—fish kept "Overnight in Tank"), consisted of bycatch individuals collected from a small shrimp trawler (outrigger trawler) and kept overnight in a confinement fisher tank (30 fish in a ~ 2000 L tank, or ~ 0.015 fish/liter), under constant flow of seawater (open circulation). Fish chosen for this distinct treatment were fish which were alive after the shrimp trawling and were in good condition. This treatment has been included to verify whether fish would recover from trawling stress and show a distinct pattern in the stress response markers chosen. It is worth mentioning that the tank used for fish maintenance in this treatment (OT) is normally used by the fisher to keep the fish fresh and bait alive before sale. After ~ 12 h, fish were anesthetized in benzocaine (60 mg/L), displaying loss of balance after 2–3 min. Blood samples were then collected by caudal puncture using heparinized syringes. Blood samples were centrifuged in a microcentrifuge (2100 rpm for 5 min). Plasma was pipetted out and frozen at − 20 °C for later analysis in the Laboratory of Comparative Physiology of Osmoregulation, Federal University of Paraná in Curitiba, Paraná. At the time of blood withdrawal, all fishes were identified to the lowest taxonomic level and their total length (cm) was measured. Weight estimates (g) were obtained at the www.fishbase.org site from the measured lengths for each specimen.

Assessment of the acute stress response to trawling

The responses to the intense and acute stress of trawling were evaluated employing the main primary stress marker in bonefish, plasma cortisol, as well as other established

secondary stress markers: plasma glucose, osmolality, chloride, and magnesium. Plasma cortisol was determined using a solid-phase immunoabsorbent enzyme assay (ELISA), based on the competitive binding principle (DRG Cortisol Elisa, Ref EIA-1887). Blood glucose was measured in situ, in a small blood sample obtained from the caudal vein, using an Accu-Chek® digital device (Roche—Performa Nano model). Plasma osmolality was determined by reading samples without dilution in a vapour pressure micro-osmometer (5520 VAPRO, Wescor®, USA). Chloride and magnesium concentrations in plasma were determined by spectrophotometry (Ultrospec 2100 PRO Amersham Pharmacia biotech, Sweden) using colorimetric kits (Labtest, Brazil), and reading absorbance, respectively, at 470 nm and 505 nm. Fish plasma samples were diluted 1:2 for the determination of Cl^- ions and 1:4 for the determination of Mg^{+2} ions.

Validation of the ELISA for plasma cortisol

Recovery and linearity tests were performed using four samples of marine-estuarine fishes, for the validation of the cortisol ELISA assay. Samples from *Sphoeroides testudineus* (sample 1), *Mugil liza* (sample 2), *Menticirrhus americanus* (sample 3), and *Genyatremus luteus* (sample 4) were selected and 54/96 wells were used from an ELISA plate. The recovery test was performed employing 1:1 dilution of the selected samples and the 50, 100, and 200 $ng.ml^{-1}$ standards. For the linearity test, serial dilutions (1/2, 1/4, 1/8, and 1/16) were made using standard 0 and the four selected samples. All tests were performed in duplicates. The precision and reproducibility of the assay (intra-assay) were verified by calculating the coefficient of variation (CV) of repeated measures of samples within the same assay. Inter-assay precision and reproducibility was determined by assaying the same samples in three separate assays, again though it's CV. The standard curve showed a high correlation coefficient between Logit OD and log concentration of standard solutions for all plates used in the assays ($R^2 = 0.9821$—plate 1; 0.934—plate 2; 0.9804—plate 3; 0.9721—plate 4; 0.9705—plate 5; and 0.9628—plate 6). The precision and reproducibility of the assay was good, CVs were all < 10%, as was the CV for duplicates of all samples (see Supplementary Material 1).

Data analyses

Given the high intra- and interspecific variability of the primary and secondary stress markers, and the fact that data came from different species, graphs were prepared as scatter plots, displaying individual values. Additional analyses are provided in the Supplementary Material 2: Kruskal–Wallis comparisons among DT, LT, and OT groups for each parameter, as well as Spearman's correlations between pairs of parameters. To verify which components (cortisol, glucose, osmolality, chloride, magnesium, length, weight, and season) best explained the variation in the stress response among individuals within each group, the Principal Component Analysis (PCA) was performed, and the groupings classified by the k-means algorithm. To evaluate the similarity of the stress response among individuals and groups, the Permutational Multivariate Variance Analysis (PERMANOVA) was performed in the "vegan" package, with the Euclidean distance measure and 999 permutations applied. This analysis allowed to verify if the physiological response of the organisms to capture stress was different between the treatments applied (DT, LT, and OT). Data analyses were performed using SigmaPlot® software version 11.0 and "R" version 3.6.1. The significance level adopted was always of 0.05.

Results

General characteristics of samples

A total of 224 samples were collected, representing 36 species from 19 families of wild subtropical marine teleosts. Fifty-nine samples were assigned to the DT group (dead after trawling), 93 to the LT group (live after trawling), and 72 to the OT group (live, kept overnight in a tank by the local fisher). See Supplementary Material 3 for the list of species sampled, and length/weight data of all specimens.

Variability of the stress response

The variability of the response among individuals and species was remarkable, within each of the three groups. For cortisol (Fig. 1), there were basically three types of responses to the intense and acute stress of capture by trawling as follows: (i) species with high response; (ii) species which presented both high and low responses, and (iii) species which presented only a low response. For example, the Atherinopsidae-brazilian silverside *Atherinella brasiliensis* presented, almost always, extremely high values, both in DT (average 1291.93 ng/mL) and in LT (average 3636.85 ng/mL), except for a single individual who had a low value in DT (89.6 ng/mL). Other species showed both low and high values for cortisol, in the DT and/or LT groups, such as the Centropomidae–sea bass *Centropomus undecimalis* (min 54 ng/mL in DT–max 160 ng/mL in DT), Paralichthyidae–bay whiff *Citharichthys spilopterus* (min 44 ng/mL in LT–max 448 ng/mL in DT), Haemulidae–stonefish *Genyatremus luteus* (min 14 ng/mL in DT– max 294 ng/mL in LT), and Mugilidae–gray mullet *Mugil liza* (min 5 ng/mL in LT–max 595 ng/mL in DT) (Fig. 1). In contrast, some individuals, mainly representatives of the family

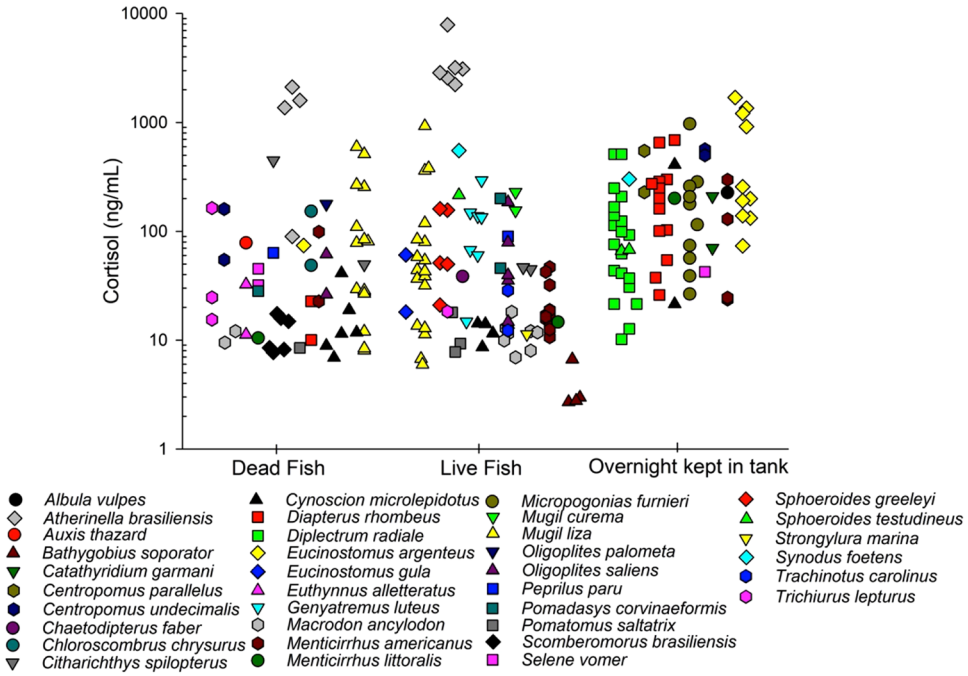

Fig. 1 Plasma cortisol (ng/mL) of the trawled fish within each group: Dead Fish, Live Fish, and Fish kept overnight in tank. Symbols with the same colors and shapes represent individuals of the same species

Sciaenidae–smallscale weakfish, king weakfish, and southern kingcroaker (respectively: *Cynoscion microlepidotus*, *Macrodon ancylodon, Menticirrhus americanus*), always had a very low cortisol response both in DT and in LT (average 14, 11, 25 ng/mL, respectively), while some individuals of the same species (*Cynoscion microlepidotus*, *Macrodon ancylodon, Menticirrhus americanus*) showed high values of cortisol only in the OT group, of ~ 297–408 ng/mL (Fig. 1).

Glucose was also shown to be highly variable among groups, species, and individuals. The minimum values (12 mg/dL) were presented by the Sciaenidae–whitemouth croaker *Micropogonias furnieri* and by the Achiridae–flatfish *Catathyridium garmani* in OT (Fig. 2). The maximum value (534 mg/dL) was measured in Serranidae—the pond perch *Diplectrum radiale*, in the same treatment. However, a few species showed less variability of this marker within a same group. For example, *Cynoscion microlepidotus* (20–68 mg/dL), the Scombridae–Spanish mackerel *Scomberomorus brasiliensis* (72–132 mg/dL) in the DT, the Tetraodontidae–green puffer *Sphoeroides greeleyi* (32–67 mg/dL) in LT, and the Gerreidae–silver mojarra *Eucinostomus argenteus* in OT (25–89 mg/dL). There was an exception of one individual of this last species, which showed a glycemia of 439 mg/dL in this treatment (Fig. 2).

Plasma osmolality levels were visually "concentrated" around 400 mOsm/kg H_2O (Fig. 3). However, there was some dispersion around this value, with some very high values (around 600–947 mOsm/kg H_2O), and a few very low ones (85–243 mOsm/kg H_2O). Very high values among these treatments were recorded for Scombridae–the frigate tuna *Auxis thazard* (803 mOsm/kg H_2O), Carangidae–the lookdown *Selene vomer* (784 mOsm/kg H_2O) and Haemulidae–the roughneck grunt *Pomadasys corvinaeformis* (816 mOsm/kg H_2O) in DT group. And for Gobiidae–the frillfin goby *Bathygobius soporator* (906 mOsm/kg H_2O), *Pomadasys corvinaeformis* (663 mOsm/kg H_2O), and *Menticirrhus americanus* (682 mOsm/kg H_2O) in LT. Relatively low values of osmolality were also found in these treatments (DT and LT), being frequent in *Cynoscion microlepidotus* (e.g. 85 and 166 mOsm/kg H_2O) and *Macrodon ancylodon* (e.g. 107 and 129 mOsm/kg H_2O). OT species presented higher variability in the osmolality values, with individuals of the same species presenting a wide range of values, within this same treatment, as especially observed for Gerreidae–the caitipa mojarra *Diapterus rhombeus* (278–947 mOsm/kg H_2O) (Fig. 3).

Accordingly, plasma chloride concentrations were also quite varied in OT individuals, mostly intraspecific variation. This was noted for *Diplectrum radiale* (range 82–401 mM) and *Diapterus rhombeus* (109–333 mM), and *Bathygobius soporator* (33–399 mM) in LT. *Pomadasys corvinaeformis* showed high Cl^- values in both DT (357 mM) and LT (192–310 mM). Some individuals showed little variation. This was the case of the Carangidae–the castin leatherjacket *Oligoplites saliens* (120–127 mM in DT, and 120–132 mM in LT), *Mugil liza* (72–161 mM in DT, and 121–185 mM in LT), *Atherinella brasiliensis* (143–161 mM in DT, and 173–208 mM in LT), and *Macrodon ancylodon*, especially in DT: 124–128 mM. Extremely low values of Cl^- were found for Scombridae, the little tunny *Euthynnus alletteratus* (34 mM), and in Trichiuridae, the largehead hairtail *Trichiurus lepturus* (40 mM), these last two in DT group (Fig. 4).

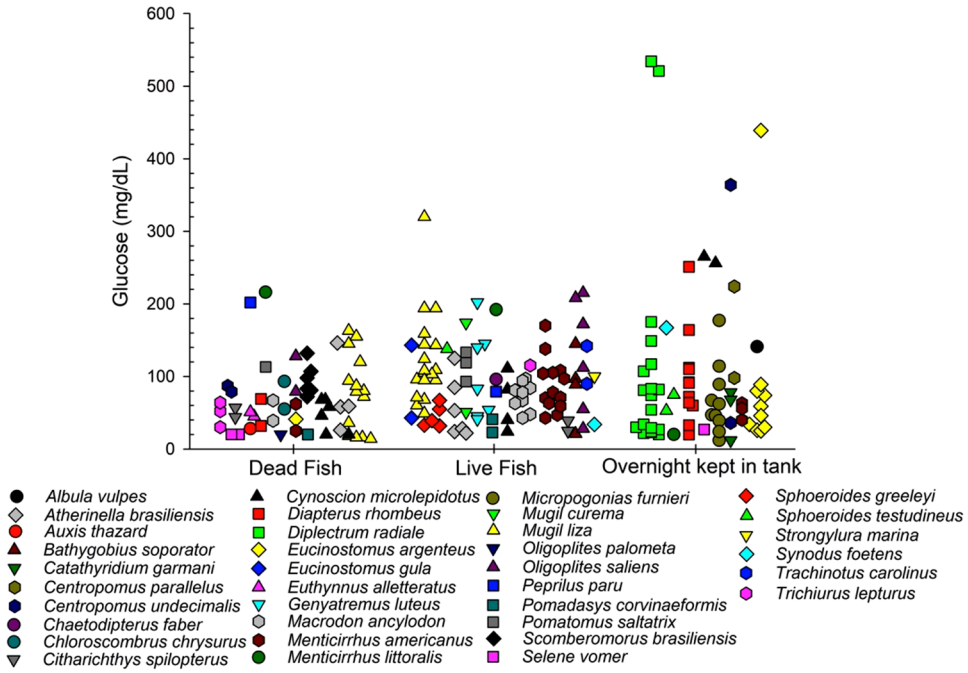

Fig. 2 Blood glucose (mg/dL) of the trawled fish within each group: Dead Fish, Live Fish, and Fish kept overnight in tank. Symbols with the same colors and shapes represent individuals of the same species

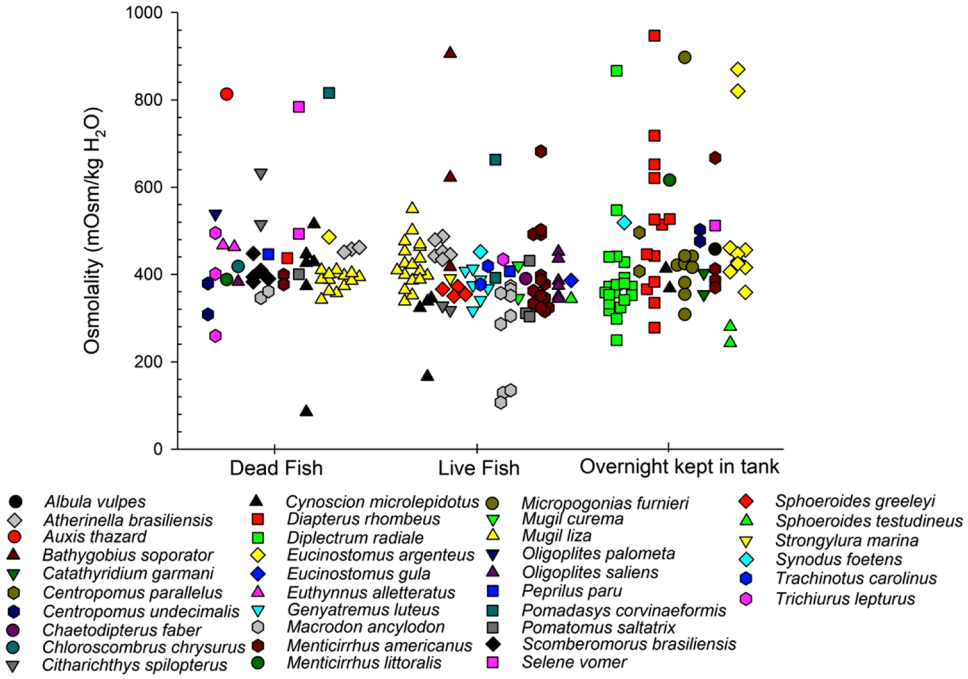

Fig. 3 Plasma osmolality (mOsm/kgH_2O) of the trawled fish within each: dead fish, live fish, and fish kept overnight in tank. Symbols with the same colors and shapes represent individuals of the same species

Plasma Mg^{2+} exhibited a modal value around ~ 1.4 mM, but showed variability and dispersion in a slightly more asymmetric way, when compared to osmolality and chloride, with more frequent very high values. There was especially great variability among OT individuals of a same species. All species sampled in this group–OT—showed a large range of variation in this ion. The greatest differences were found in *Diapterus rhombeus* (1.4–9.7 mM), *Micropogonias furnieri* (1.8–10.3 mM), and *Eucinostomus argenteus* (1.9–8.9 mM). Great variability in magnesium concentration was also noted in *Cynoscion microlepidotus* in DT (0.02–11 mM). Considerably lower and less variable values were found in individuals of the species *Mugil liza* in DT (0.6–2.4 mM), and LT (0.2–2.0 mM) groups, and *Scomberomorus brasiliensis* (1.7–2.1 mM), and *Sphoeroides greeleyi* (1.7–1.9 mM) in DT group. *Atherinella brasiliensis* presented higher concentrations of this ion

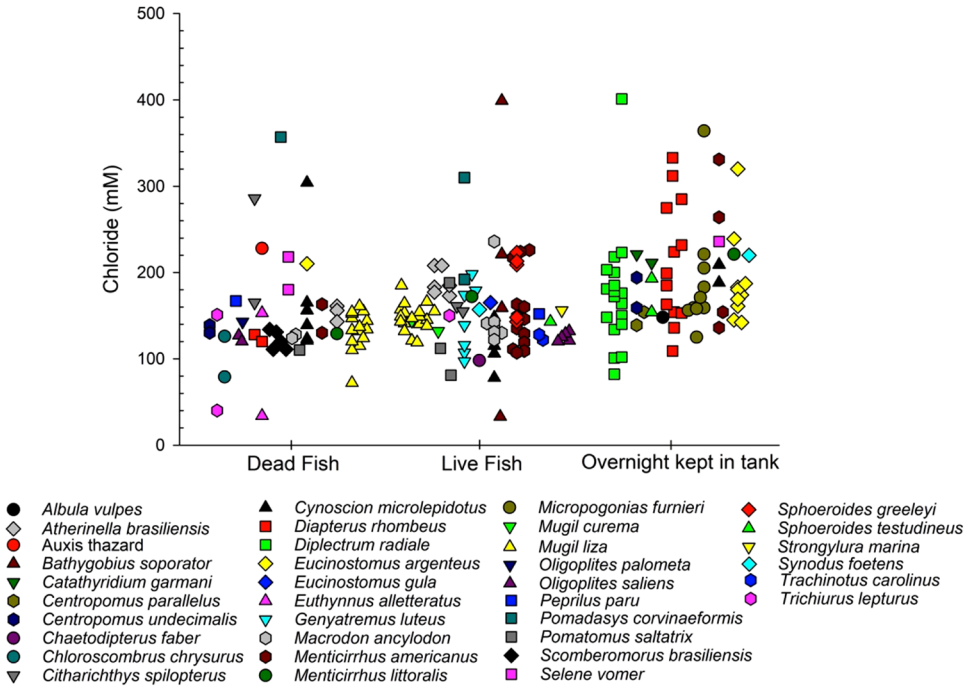

Fig. 4 Plasma chloride (mM) of the trawled fish within each: dead fish, live fish, and fish kept overnight in tank. Symbols with the same colors and shapes represent individuals of the same species

among the sampled species in DT (6.7–12 mM), and high concentrations in LT (5.3–9.5 mM) (Fig. 5).

Intra- and inter-specific variability in the acute stress response

Given the non-controlled and biased character of this "natural experiment", we applied a PCA analysis on the obtained raw data provided above, to group individuals/species based on the similarity of the pattern of their stress response. The analysis allowed us to determine which physiological components better explained the observed variation. Plasma cortisol (Comp.1) and osmolality (Comp.2) components together explained more than 67% of the variance found within each group, being 73.8%, 67.5%, and 67.2% of the variability of data in groupings DT, LT, and OT, respectively

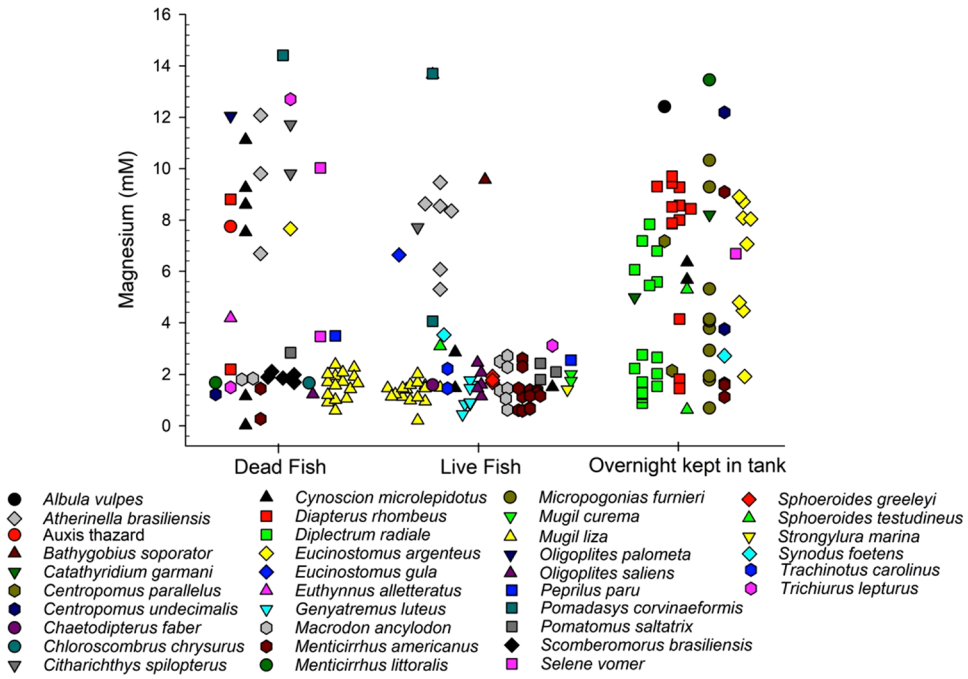

Fig. 5 Plasma magnesium (mM) of the trawled fish within each group: Dead Fish, Live Fish, and Fish kept overnight in tank. Symbols with the same colors and shapes represent individuals of the same species

(Figs. 6 A, B, and C). From these results we can suggest the formation of four clusters—black, red, blue, and green symbols (dots surrounded by the ellipses). The first cluster (black circles) displays individuals which presented distinctly high levels of cortisol and magnesium after stress, represented only by *Atherinella brasiliensis* (both in DT and LT treatments). The second grouping was comprised of individuals which presented high osmolality and low glucose values (red circles). The third (blue circles) and fourth (green circles) groupings included individuals which had low-intermediate levels of cortisol with slight variations in secondary stress responses. These clusters (blue and green) include species from several teleost fish families, without any evident trend regarding habitat use among them.

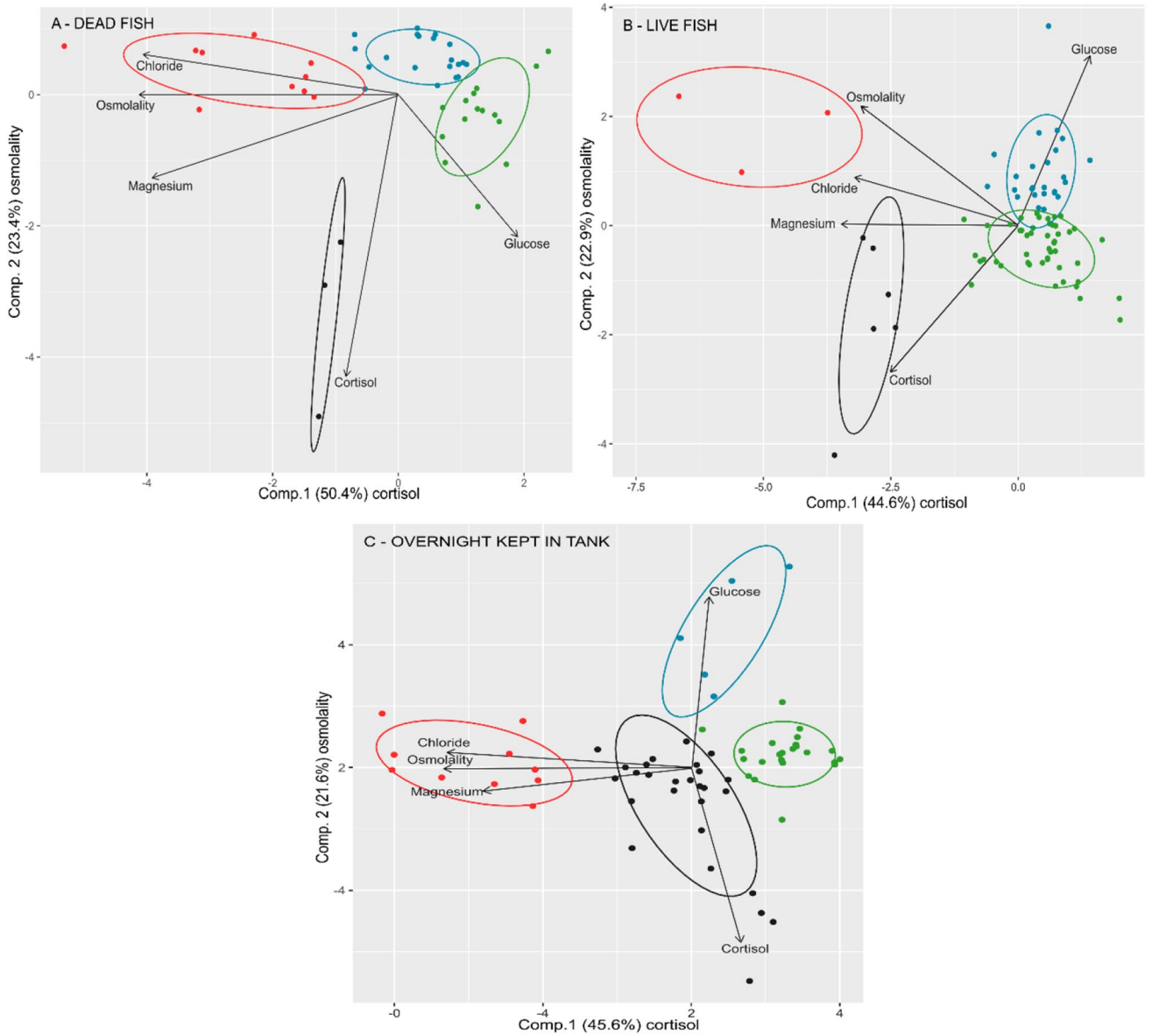

Fig. 6 Scatterplots of PCA applied to physiological parameters cortisol, osmolality, glucose, chloride, and magnesium. Graphical result of the analysis for Dead Fish (**A**, DT), Live Fish (**B**, LT), and Fish kept overnight in tank (**C**, OT). The first two components (Comp.1 = cortisol; Comp.2 = osmolality) accounted for 73.8%, 67.5%, and 67.2% of the variability of data in groups DT, LT, and OT, respectively. These results identified four different ways of coping with stress, within each group: represented by the dots and ellipses: red, high osmo-ionic concentrations and low cortisol and glucose; black, high cortisol and high magnesium (DT and LT) or variable cortisol and low osmo-ionic levels (OT); blue/green, lower levels in general, or elevated glucose. The analysis also revealed a positive linear correlation between cortisol, osmolality, chloride and magnesium components; and a negative correlation (exception DT) between cortisol and glucose. Fish length, weight, and season components together explained less than 10% of the data variation and were, therefore, excluded from the analysis (see Supplementary Material 2)

The tightness of the specific response patterns found in the present study are reflected by the reproducibility of the responses between individuals of *A. brasiliensis* (black circles, DT and LT), *M. liza*, *S. brasiliensis*, *G. luteus*, the cyanides (*M. ancylodon*, *M. americanus*, *M. littoralis*, *C. microlepidotus*), and some carangids (*T. carolinus*, *O. saliens*), even when individuals were either dead or alive after trawling (green and blue circles). The type of response to stress presented in individuals of *A. brasiliensis* was quite peculiar, with high levels of cortisol and magnesium and no marked variation in chloride and osmolality values. The individuals belonging to the cluster of blue and green circles apparently showed great capacity to maintain osmoregulatory balance, both in individuals with low response and in individuals with high response to cortisol. *M. liza*, *S. brasiliensis*, *G. luteus*, *T. carolinus*, and *O. saliens* are examples of species that maintained osmoregulatory stability both in individuals with high and low cortisol responses. And *M. ancylodon*, *M. americanus*, *M. littoralis* and *C. microlepidotus* were examples of species that also showed maintenance of osmoionic balance, however, with low responses to cortisol. The individuals represented by the red circles were not able to cope with this type of stressor. *A. thazard*, *P. corvinaeformis*, and *B. soporator*, for example, presented osmoregulatory rupture after the capture stress, evidenced by the difficulty in remaining hyposmotic, with low cortisol and glucose values. Differently, the black cluster in OT gathers species which displayed great intra-specific variability in cortisol, also reaching very high values, but relatively stable osmolality and chloride (such as *E. argenteus*, *D. radiale*, *M. furnieri*), and occasionally high magnesium (*E. argenteus*, *D. rhombeus*, *C. undecimalis*, and *C. paralellus*).

The PERMANOVA results (performed to verify the influence of the proposed groups on data variability) showed that data variation was better explained by the inherent characteristics of the individuals (different stress response abilities) than by the association to any of the 3 groups (Table 1, Fig. 7).

Discussion

General characteristics of samples

The individuals studied here were obtained directly from small scale fishing resources in subtropical regions of Brazil. Thus, the results reflected the seasonality (April 2017–July 2018) and selectivity of the fishing gear in terms of the collected species, through random sampling. In addition, it must be mentioned that no attempts were made for determinations

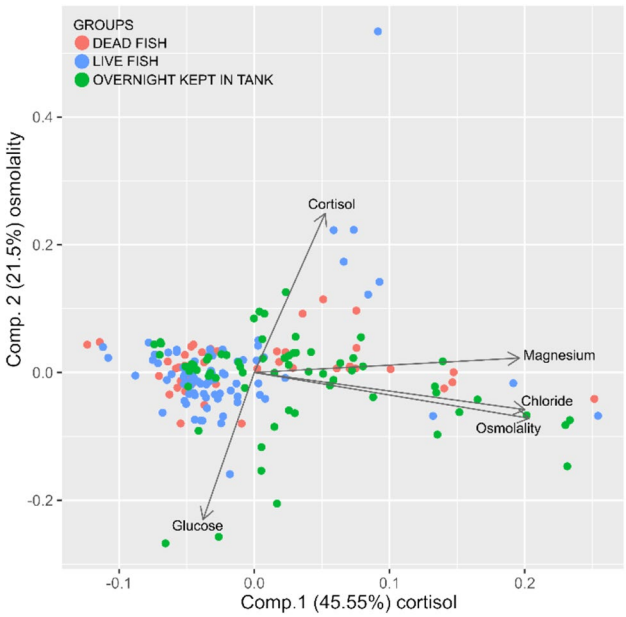

Fig. 7 Permutational multivariate analysis of variance (PERMANOVA) results applied to the physiological parameters measured. Colors refer to the 3 groups: Dead Fish, Live Fish, and Fish kept overnight in tank, with a total $n = 224$ individuals. These results showed that the differences found in the response to the capture stress were not related to the different groupings proposed, being the variability of the response to stress (way of coping with stress) specific to each individual and/or species. Besides a positive linear correlation between cortisol, osmolality, chloride, and magnesium components; there was a negative correlation between cortisol and glucose components. The analysis was performed using the "vegan" package, the Euclidean distance was measured, and 999 permutations were applied.

Table 1 Permutational multivariate analysis of variance (PERMANOVA) table for the stress response among individuals and groups: dead or live fish, and fish kept overnight in a tank)

Source of variation	df	Sum of squares	Mean of squares	F.Model	R^2	Pr (> F)
Individuals	35	5.87	0.16	4.42	0.43	**0.001**
Groupings	3	0.45	0.22	5.93	0.03	**0.001**
Residuals	186	7.06	0.03		0.52	
Total	224	13.38				

Bold print indicates significant results

of basal levels of the parameters assayed. Rather, an exploratory analysis was here conducted, to investigate patterns that might represent different types of ways of coping with acute stress from subtropical wild fishing resources.

Measures of physiological stress have the potential to contribute to the identification and assessment of complex challenges to wildlife, aiming at conservation and management (Baker et al. 2013). Physiological research in wild populations provides basic data, allowing fast evaluation of natural and anthropogenic pressures, helping to elucidate causal mechanisms and assess the effectiveness of targeted actions (Cooke and Connor 2010; Baker et al. 2013). There are several examples of applications of stress measures, such as in the fishing and release activities and on the sensitivity of bycatch species (Cook et al. 2015, 2018; McLean et al. 2016; Schlenker et al. 2016). Another major application is on the evaluation of physiological requirements and limitations of cultivated species or species with potential for aquaculture (Fevolden et al. 1991; Barnett and Pankhurst 1998; Froehlich et al. 2016). Studies on stress of wild animals also allow the determination of which species currently live closer to their upper thermal tolerance limits, which physiological systems constrain these limits, and how species differ in acclimatization capacity, potentially broadening their ranges of thermal tolerances (Somero 2010). Finally, these studies are very useful for incorporation of physiological data into mechanistic models to determine how natural and anthropogenic stressors can affect organisms and ecosystems (McKenzie et al. 2016; Birnie-Gauvin et al. 2017).

No influence of treatments: PERMANOVA analysis

Although the initial idea was to evaluate the response to capture stress in three different treatments (Dead Fish–DT, Live Fish–LT, and Fish kept Overnight in Tank–OT), the PERMANOVA analysis indicated that there was no influence of the proposed treatments on the physiological variability found. The addition of the OT treatment was done to verify whether it would be possible for the fish to recover from trawling stress after this period. If the type of response of several species in DT and LT groups are compared, similar responses between individuals of the same species are observed. It can be concluded that the live fish after trawling were in the same stressful condition as the dead individuals of the same species. This happened, for example, for *Mugil liza*, *Atherinella brasiliensis*, the cyanidae (*M. ancylodon*, *M. americanus*, *M. littoralis*, *C. microlepidotus*). When performing two-groups comparison (Mann–Whitney tests, dead × live fish), using all those species, and their respective values, the result is that the same species showed similar values for the markers, whether dead or alive after trawling: $P = 0.574$ for cortisol, $P = 0.598$ for osmolality, $P = 0.151$ for chloride, and $P = 0.104$ for magnesium, $n = 82$ for each parameter. Thus, the most striking physiological variability found refers to the different types and/or strategies for coping with stress among the various species collected, independent of being alive or dead after trawling or remaining overnight in a seawater tank. Fish capture by trawling, followed by transfer and maintenance of the subjects for ~ 12 h in the tank (OT treatment), did not allow individuals to recover their putative pre-stress levels of primary and secondary stress markers. Fish in the OT group tended to display an elevated stress response rate for both cortisol and the secondary markers, when compared to the other two groups. Other factors that possibly favored the development of stress in this treatment were the confinement regime (~ 2000 L), with hierarchically distinct species in a limited space (Marino et al. 2008; Fischer and Romero 2018), and even the removal of the fish from the tank for sampling (Ellis et al. 2004).

Stress responses must be compensatory and adaptive (eustress) to allow the animal to overcome the threat. However, stress responses may lose their adaptive meaning (distress) under chronic or intense acute stressors and this may result in adverse effects on growth and reproduction (Samaras et al. 2018) and in dysregulation or suppression of immune function (Tort 2011). In fact, the "fight-or-flight" response is certainly not regulated the same way in all species of fishes (Balasch and Tort 2019). In response to a stressor, animals may exhibit distinct physiological and behavioral responses (i.e., McEwen and Wingfield 2003; Romero et al. 2009). These responses may be consistently different between individuals (due to personality type) but are putatively stable within an individual of a certain age. Glucocorticoid release rates may be conserved in proactive and reactive stress coping styles among diverse species, but may depend on contexts such as foraging, or daytime (Balasch and Tort 2019; Wong et al. 2019). However, here, we have examined the acute response of the fish to the extreme and essentially lethal stress of trawling. There was no evaluation of any putative chronic or late effects of the stress imposed on these fish. In this way, the discussion below will emphasize the differences in patterns of response to the acute capture stress, as pointed out by the PCA (formation of four clusters of individuals), regardless of the group ascribed to the fish.

Integrating the several stress markers evaluated: the multivariate analyses (PCA)

The PCA revealed distinct physiological consequences in the fish submitted to a same intense and acute stress. These analyses also showed that weight and length (intrinsic factors) and season (extrinsic) did not significantly affect the results. Likewise, levels of plasma cortisol after capture and handling stress across six species of freshwater fish (largemouth bass *Micropterus salmoides*, smallmouth bass *Micropterus*

dolomieu, rock bass *Ambloplites rupestris*, bluegill *Lepomis macrochirus*, pumpkinseed *Lepomis gibbosus*, and northern pike *Esox lucius*) were also not correlated with fish length (Lawrence et al. 2018). Intrinsic factors such as life stage, life history or reproductive stage, and sex, as well as extrinsic factors such as climate, competition, food resources, habitat quality, social structure, parasitic load and injury, human disturbances, interaction with predators, and handling are exhaustively cited as influencers in the response of glucocorticoids to a specific stress, in a variety of vertebrates (Iwama et al. 2004; Busch and Hayward 2009; Madliger et al. 2015, 2018; Birnie-Gauvin et al. 2017). However, a cause-and-effect relationship is not always very clearly described (Mommsen et al. 1999; Romero et al. 2009), and multiple interactions probably result in complex and variable responses. All these non-controlled parameters may have influenced the variability in the response concerning mortality, and levels of cortisol and glucose and osmolality, chloride and magnesium measured in our samples, particularly in this type of experimental design.

Cortisol

Our results evidenced the existence of inter-specific differences in the magnitude of the cortisol release/response after severe acute stress (Iwama et al. 2006; Kalamarz-Kubiak 2018). Some species had always high levels of cortisol, others showed frequently low levels of cortisol, while others showed both high and low levels in response to the same stressor. *Atherinella brasiliensis* (Atheriniformes, Atherinopsidae) presented extremely high values of cortisol in this study, a feature that was repeated for all sampled individuals of this species. The pattern was the same for specimens sampled in different months and for individuals either dead (DT) or alive (LT) after trawling. Very high values of plasma cortisol for this same species (~ 564–650 ng/mL) have been previously reported for individuals caught in Paranaguá Bay 7 months after the Vicuña oil spill (Souza-Bastos and Freire 2011). Values as high as those assayed in *A. brasiliensis* have also been reported for the chub *Squalius cephalus* (Cypriniformes, Cyprinidae): the highest individual level was 1927 ng/mL, mean levels were of 1500 ng/mL, after intense acute handling stress (Pottinger et al. 2000). The potentially adverse effects of supposedly high circulating levels of cortisol found both at rest (50–100 ng/mL) and under conditions of stress in *S. cephalus* were proposed to be offset by the lower affinity of the cortisol receptor, rather than the low abundance of target-tissue receptor sites (Pottinger et al. 2000). Recurrent high cortisol levels after stress were found for the sea bass *D. labrax* (~ 600–800 ng/mL) in the literature (Rotllant et al. 2003; Fanouraki et al. 2011; Samaras and Pavlidis 2018), and they were explained by increased activity of the interrenal cells (Rotllant et al. 2003).

In the present study, most species of demersal habits, such as the Perciformes Sciaenidae (*C. microlepidotus*, *M. ancylodon, M. americanus, M. littoralis*), and Gobiiformes Gobiidae (*Bathygobius soporator*) displayed very low levels of plasma cortisol after the trawling. It is possible that plasma cortisol in these fishes would later increase. In fact, a delayed cortisol response was reported for the Scorpaeniformes, Hemitripteridae—sea raven *Hemitripterus americanus* (Vijayan and Moon 1993): this fish showed a slow response in post-stress cortisol levels, taking up to 4 h to reach a peak after acute stress. A delayed cortisol production may be a feature conserved in some families of fish due to trade-offs in neuroendocrine mechanisms and regulation, particularly CRF, and ACTH release, or else due to the involvement of alternative pathways and other hormones in the stress response (Vijayan and Moon 1993; Kalamarz-Kubiak 2018). Indeed, plasma cortisol of fish from the family Scianidae (*C. microlepidotus*, *M. ancylodon*, *M. americanus*, *M. littoralis*) in the present study was only elevated in the OT treatment. This altered stress response may represent an adaptation to avoid over-mobilization of energy in a species with inactive lifestyle and low metabolic activity (Vijayan and Moon 1993; Kalamarz-Kubiak 2018). Plasma cortisol concentration is probably not a direct determinant of osmoregulatory mechanisms after acute stress in the Sciaenidae, and it is necessary to investigate the other mechanisms that support this type of response. Significantly reduced corticosteroid responses (both baseline and after stress) were also related to chronic stress in birds (*Sturnus vulgaris*). Under chronic stress, the hypothalamus regulates arginine vasotocin (AVT) release, which putatively leads to inhibition of adrenocorticotropic hormone (ACTH) release, leading to lower concentrations of stress-induced corticosterone (Rich and Romero 2005). In fact, not only do some fish display a low cortisol response to stress, but there is evidence that in some fish, other hormones and neuro-mediators are also involved in the response to stress, such as AVT, isotocin, urotensis, dopamine, serotonine or endorphin (Kalamarz-Kubiak 2018).

Cortisol data after capture stress in teleost fish reported in the literature are also quite variable among the species sampled and the magnitude of the response; plasma cortisol usually increases along the duration of capture, handling, and exposure to air. Previous data in the literature in general refer to cultivated fish or planned experiments on handling of fishes that lead to no mortality, in contrast to the extreme conditions of long trawling of wild fish that is reported here, and which led to mortality. Peak levels and time courses of cortisol change are variable, as also are the protocols of time and types of stress imposed. For example, plasma cortisol peaks (158–208 ng/mL) in the jundiá *Rhamdia quelen* occurred only 1 h after the exposure to the

stress of capture and transfer from the tank. Afterwards, cortisol values decreased progressively but did not return to basal levels even after 24 h (Barcellos et al. 2001). Juvenile wild flounders *Paralichthys orbignyanus* caught with a trawl and immediately sampled after capture (~ 3 min) presented cortisol levels indicative of so-called pre-stress conditions (~ 2.56 $ng.g^{-1}$); these levels increased 6.7-fold after 1 h of transport, but returned to low basal levels after 24 h (Bolasina 2011). Inter-specific variability when six species of wild freshwater fish were submitted to the capture stress of angling was noted by Lawrence et al. (2018). However, these authors could determine that cortisol levels in blood samples obtained in the first 2–4 min after capture would be consistently low, representing basal pre-stress levels (Lawrence et al. 2018). After these initial minutes post-capture, cortisol increased, in level and intra-specific variability. For example, after 6 min reaching 100–300 ng/mL in the bluegill *Lepomis macrochirus*, or after 4 min reaching 50–250 ng/mL in the pumpkinseed *Lepomis gibbosus*, or else remaining below 60–100 ng/mL in the rock- or largemouth basses (Lawrence et al. 2018). Thus, these studies found the general pattern of low levels of plasma cortisol in samples taken in the first 2–3 min post-capture, tending to a later rise. Interestingly, Weissman et al. (2018) reported behavioral (reflexes) indications of stress as dependent on the duration of air exposure and towing in wild monkfish *Lophius americanus* captured along with sea scallops; a "cryptic" cortisol response was observed, in which plasma cortisol remained < 10–20 ng/mL, like the pattern observed here in some species belonging to the red or blue or green clusters (Weissman et al. 2018). Also in wild fish, the white marlin *Kajikia albida*, captured by angling in recreational fisheries, the duration of angling time was directly related to sodium, chloride, magnesium, and cortisol (Schlenker et al. 2016). Cortisol levels in the marlin reached 200–400 ng/mL (Schlenker et al. 2016), compatible with the fishes in the black cluster in our study, showing high cortisol levels, and increases in plasma osmo-ionic parameters, perhaps indicating disturbance of salt secreting mechanisms during this intense acute stress of capture.

In our study, besides this notable inter-species variability, some species showed marked intra-specific variability in post-trawling plasma cortisol levels, with some individuals of the same species showing very high values, while others displayed low values. This happened, for example, with the Pleuronectiformes flounders Achiridae, *Catathyridium garmani* and Paralichthyidae, *C. spilopterus*, the Mugiliformes, Mugilidae *M. liza*, and the Perciformes, the Gerreidae *E. argenteus* and *D. rhombeus*, as already demonstrated in other studies for other species. For example, the European sea bass *Dicentrarchus labrax* had individuals displaying the so-called low-(LR) and high-(HR) responses of cortisol after exposure to acute stressors. Different LR and HR responders were also reported in cods *Gadus morhua* when subjected to handling and thermal stress (Hori et al. 2012), and in the seabream *Sparus aurata* submitted to handling and confinement for a period of 3 h (Tort et al. 2001). This pattern may explain the high intra-specific variability in some of the species studied here.

Glucose

Increased plasma glucose levels after stress are widely reported (Iwama et al. 2006). However, plasma glucose and hepatic glycogen concentrations vary substantially depending on the species, stage of gonadal maturation (Wendelaar-Bonga 1997) and metabolic status of the animal (Mommsen et al. 1999). The enormous variability in glucose levels was evident in fish from all clusters, not proving to be a good stress marker in studies of this type—"not controlled" in a natural environment. The use of glucose as a stress marker shows better results in studies with cultivated species, usually showing a significant correlation with cortisol levels (Pottinger et al. 2000; Trenzado et al. 2003). This result is to be expected, given that fish receive the same treatments and diet in aquaculture practices. However, the positive and significant correlation between glucose and cortisol was detected in wild freshwater fishes submitted to capture by angling: in largemouth bass *Micropterus salmoides*, northern pike *Esox lucius*, pumpkinseed *Lepomis gibbosus*, and bluegill *Lepomis macrochirus*. Moreover, blood glucose concentrations gradually increased with time post-capture (up to 12 min) in most species studied (Lawrence et al. 2018). In sturgeons submitted to simulated fisheries stressors, glycemia increased in fish exposed to 15 min of exercise and partial air exposure, compared to non-stressed fish. These fish showed a relatively low response for both glucose and cortisol (McLean et al. 2016). Variable blood glucose concentrations in fish may also reflect the disparity in glucose mobilization between fish with divergent low (LR) and high (HR) adrenocorticotropic responses to stressors, perhaps linked to differential activation of glycogenolytic pathways (Trenzado et al. 2003). Thus, results were also as expected, confirming previous information from the literature. The whole set of data was so variable, that cortisol and glucose did not show a significant correlation (Spearman coefficient − 0.0733, $P = 0.313$, Supplementary Material 2).

Osmolality, chloride, and magnesium

The combined actions of cortisol between glucocorticoid and mineralocorticoid receptors can be viewed as homeostasis/allostasis mediators, regulating hydromineral balance and energy metabolism. See reviews in Wendelaar-Bonga (1997); Reid et al. (1998); McEwen (2000); Korte et al. (2005); Iwama et al. 2006; Takey and Loretz 2006; McEwen

and Wingfield (2010), and Pankhurst (2011). The release of cortisol during stress can cause osmoregulatory perturbations (Wendelaar-Bonga 1997) due to changes in branchial permeability to water and electrolytes: and cortisol has a recognized role in stimulating salt secretion (hypo-osmoregulatory capacity) in marine fish (Mommsen et al. 1999; McCormick 2001; Iwama et al. 2006; Takey and Loretz 2006). In addition, cortisol has a substantial physiological impact on ion uptake in many teleosts, but this function has not been fully elucidated due to the emphasis on the role of cortisol in branchial salt secretion (Takei and McCormick 2012).

Marine teleosts hyporegulate their internal salt concentrations and osmolality at levels well below (370–480 mOsm/kg H_2O) that of seawater (~ 1050 mOsmol/kg H_2O for 34–35 psu seawater, Marshall and Grosell 2006; Freire and Prodocimo 2007, Evans and Claiborne 2009). They display very low osmotic permeability, consistently ingest seawater, absorb salt and water through the gastro-intestinal tract, and finally secrete salt (NaCl) through their gills; divalent ions such as Mg^{2+}, and SO_4^{2-} are mainly hyporegulated by the kidneys (Marshall and Grosell 2006; Freire and Prodocimo 2007; Evans and Claiborne 2009; Takei and McCormick 2012). Cortisol release promotes monovalent ion extrusion by the gills in marine species and, consequently, a lower osmolality value in the high-cortisol responders could be expected. High osmolality responders would correspond to low-cortisol responders and vice versa (Tort et al. 2001). This pattern was found in the red cluster individuals, which showed high values of chloride and magnesium and, consequently, high osmolality, presenting both low response to cortisol and low glucose concentrations. Some of these individuals are voracious predators and/or have a very active lifestyle, such as *A. thazard*, *E. alletteratus*, *P. corvinaeformis*; thus, the low post-stress glucose levels were probably due to intense mobilization of energy reserves after stress.

Osmoregulatory disorders were also noted in individuals which responded with high cortisol release, such as fish of the OT group which showed a considerable increase in chloride and magnesium concentrations (such as Gerreidae *Eucinostomus argenteus*, *Diapterus rhombeus*, and Centropomidae *Centropomus parallelus*, *Centropomus undecimalis*). Increased concentration of Cl^- ions after stress has been reported for the sea bass *Dicentrarchus labrax* due to crowding and confinement: serum Cl^- increased by ~ 8% (Marino et al. 2008). A similar result was reported for the salmon *Salmo salar*: 10% increase in Cl^- concentration, along with 76% increase in glycemia in the crowded group (Gatica et al. 2010). While capture stress mildly affected plasma osmolality and chloride in the pacu *Piaractus mesopotamicus* (Abreu et al. 2009), simulated fisheries stressors (exercise and partial air exposure) to the sturgeon *Acipenser transmontanus* for 15 min caused elevated osmolality and chloride, like what was seen for the fishes in the red cluster here (McLean et al. 2016).

Increased magnesium concentrations after exposure to a stressful load have already been observed in other studies with other fish species. This also happened here, in live fish, especially with *A. brasiliensis*. Other species of dead fish (group DT) also showed very high magnesium in plasma, perhaps indicating cell lysis. The high intra-specific variability within the OT group was also clear for magnesium, even though these fish were alive, for example in *D. radiale*, *D. rhombeus*, *M. furnieri*, and *E. argenteus*, with many values in the range 4–10 mM. Increased magnesium as a sign of stress has been reported for juveniles of the Nile tilapia *Oreochromis niloticus*, after 4 and 24 h of transport at three densities, increasing to ~ 6–16 mM (Moreira et al. 2015). And in *Salmo salar* immediately after transport and transfer to seawater, an increase in plasma Mg^+ was observed, compared to pre-stress levels: from 0.9 to ~ 6 mM. In this same study, it was found that sedated salmon returned to pre-stress levels in 72 h, with non-sedated individuals showing no recovery even one week after transport (Iversen et al. 2009).

Conclusions

A fair variety of wild coastal teleosts was sampled through artisanal fisheries trawling, and displayed a highly variable stress response, with great inter- and intra-specific variability. Four approximate "types of response" could be identified with the markers used here: species that had high cortisol and magnesium levels or widely variable cortisol and low osmo-ionic levels (black clusters); species with high osmo-ionic concentrations and low cortisol and glucose (red clusters); and species that displayed lower levels of all markers in general, or elevated glucose (blue/green clusters). The use of primary and secondary markers was adequate to contemplate the variability of responses between species. For future studies it is suggested that the physiological/genetic basis of this variability be investigated, identifying the neuroendocrine and molecular mechanisms that regulate the different responses in different species, also for conservation, better practices in fisheries, and aquaculture purposes. Finally, we suggest the use of ionic assays as alternative proxies to assess the variability in the physiological response to stress in fish, as they are relatively inexpensive and highly correlated, in diverse patterns, with plasma cortisol levels.

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Author contributions DB obtained the fish with the fisher, sampled, identified, assayed all markers except for blood osmolality, in all the fish, analysed all data she generated, and wrote the complete first version of the manuscript; CAF supervised the study, had the original idea, assayed all samples for osmolality, intensely discussed the data with DB and revised the manuscript.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declaration

Conflict of interest Both authors declare that they have no conflict of interest.

Ethical approval This study involved monitoring artisanal fisheries in two subtropical regions, as well as sampling a blood aliquot from some of the animals caught. The study protocol was approved by the Animal and Federal Experimentation Ethics Committee University of Paraná, on the use of benzocaine and blood withdrawal from teleosts (certificate #1056/2017). Fisher provided their informed consent verbally for the collection of animal blood, as well as for research-related issues. The collection of blood samples was carried out by a marine biologist researcher who adhered to animal welfare regulations and guidelines and took the course on Animal Manipulation and Experimentation required by the Federal University of Paraná.

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