oceans

MDPI

Article

Integrated Multi-Biomarker Responses of Juvenile Zebra Seabream (*Diplodus cervinus*) to Warming and Acidification Conditions

Marta Dias [1,2,3,*], Pedro Pousão-Ferreira [4], Mário S. Diniz [1,2], António Marques [4,5], Rui Rosa [3,6], Patrícia Anacleto [3,4] and Ana L. Maulvault [1,2,4]

1 UCIBIO—Applied Molecular Biosciences Unit, NOVA School of Science and Technology, NOVA University of Lisbon, Campus de Caparica, 2829-516 Caparica, Portugal
2 Associate Laboratory i4HB Institute for Health and Bioeconomy, NOVA School of Science and Technology, NOVA University of Lisbon, 2829-516 Caparica, Portugal
3 MARE—Marine and Environmental Sciences Centre & ARNET—Aquatic Research Network, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal; panacleto@ipma.pt (P.A.)
4 IPMA, I.P., Portuguese Institute for the Sea and Atmosphere, I.P., Division of Aquaculture, Upgrading and Bioprospection, Av. Doutor Alfredo Magalhães Ramalho 6, 1495-165 Lisbon, Portugal
5 CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos S/N, 4450-208 Matosinhos, Portugal
6 Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal
* Correspondence: maddias@fc.ul.pt

Abstract: The impacts of climate change-related stressors are becoming more noticeable in the ocean, particularly in coastal marine ecosystems. Yet limited information still exists on the physiological state and ecological resilience of marine fish species, especially during their early life stages (i.e., larvae and juveniles). The present study investigated the effects of chronic exposure to seawater warming (OW; $\Delta T = +4$ °C) and acidification (OA; $\Delta pH = -0.3$ pH units, equivalent to pCO_2~1000 µatm), acting alone or combined (OWA), on juvenile zebra seabream (*Diplodus cervinus*) physiological resilience, considering distinct levels of biological organization (i.e., biochemical, cell, organ and individual levels). After 60 days of exposure, both stressors, in isolation or combination, significantly decreased specific growth rate (−11% in OW, −42% in OA and −49% in OWA) and leukocyte counts (from −29% in OA and OWA up to −37% in OW) in relation to the control treatment. In addition, a decreased Fulton's condition index (K) was observed under warming and acidification in combination (−35% in OWA). At the cell level, OW, OA and OWA triggered different biomarker responses in *D. cervinus* (i.e., up-regulation, down-regulation, or absence of significant effect). In general, the results are suggestive of an antagonistic effect when warming and acidification are combined. OWA yielded the highest integrated biomarker response (IBR) index value in the whole organism, muscle, brain and gills of *D. cervinus* juveniles, therefore suggesting that the effects of these stressors are more severe when they act together. The distinct patterns observed in each stress scenario highlight the importance of carrying out further studies adjusted to the specificities of different regions, i.e., accounting not only for the type and degree of severity of environmental stressors already felt and/or projected for that specific area, but also the physiological plasticity of species that inhabit a particular ecosystem. The gathered knowledge will allow one to determine the vulnerability of particular marine species and geographic areas and, most importantly, to draw up effective and tailor-made conservation strategies to overcome climate change impacts.

Keywords: marine fish; animal fitness; integrated biomarker response; climate change; oxidative stress; blood cell counts

Citation: Dias, M.; Pousão-Ferreira, P.; Diniz, M.S.; Marques, A.; Rosa, R.; Anacleto, P.; Maulvault, A.L. Integrated Multi-Biomarker Responses of Juvenile Zebra Seabream (*Diplodus cervinus*) to Warming and Acidification Conditions. *Oceans* **2024**, *5*, 571–590. https://doi.org/10.3390/oceans5030033

Academic Editor: Beatriz Morales-Nin

Received: 26 May 2024
Revised: 8 July 2024
Accepted: 30 July 2024
Published: 7 August 2024

1. Introduction

The increasing release of anthropogenic carbon dioxide (CO_2; ~+40% since pre-industrial times) has notoriously affected marine ecosystems over many regions of the world, leading to increased seawater surface temperatures (due to so-called "global warming") and to reduced seawater surface pH levels due to "ocean acidification" [1,2]. Such effects are expected to become aggravated in the future, with the most recent projections of the Intergovernmental Panel for Climate Change report (SSP5-8.5; [2]) pointing out to an average seawater pH decline of 0.3–0.4 pH units and an average temperature increase of 4.4 °C, until the end of this century.

These changes in seawater abiotic conditions could play a preponderant role in the ocean's productivity and biodiversity, particularly affecting shallow coastal areas subjected to strong hydrographic alterations and anthropogenic pressures (e.g., estuaries and coastal lagoons). Such impacts on marine ecosystems' structure and balance can potentially translate into lower global seafood production, from both wild fisheries and aquaculture [3]. Over the last years, researchers have devoted considerable attention to the effects of climate change-related stressors in marine ecosystems. However, given the novelty and complexity of the topic, there is certainly a need to study stressors' interactions, their potential effects at different biological organization levels (e.g., whole organism, tissue, cellular) being of particular importance. Marine organisms can tolerate environmental variability to some extent through acclimation, though this process is energetically expensive and dependent on species, genetics, and thermal histories [4–6]. When a given stressor negatively impacts an organism, a cascading sequence of biological events is triggered, to activate a fast and effective response to reorganize energy resources [7]. The most immediate responses to stress will occur at lower biological levels (molecular and cellular), eventually escalating to alterations in species growth and fitness—i.e., at the whole organism level—and, subsequently, to changes at the population and community levels [8].

Zebra seabream (*Diplodus cervinus*) is a commercially and ecologically relevant marine fish species, inhabiting temperate waters in the Eastern Atlantic Ocean, including the Mediterranean Sea [9]. This species has a wide latitudinal distribution, inhabiting waters ranging between 12.5 and 24.7 °C [9–12]. Hence, different populations can be exposed to distinct maximal habitat temperatures and consequently have a particular critical thermal maximum (CTMax) [13,14]. In recent years, this species has gained attention within the scientific community, both from an ecological and aquaculture production point of view, due to overfishing and the compromised recruitment of wild stocks [10]. Thus, the aim of the present study was to understand whether chronic exposure (i.e., 60 days) to seawater temperature (OW; $\Delta T = +4$ °C) and acidification (OA; $\Delta pH = -0.3$ pH units, equivalent to pCO_2~1000 µatm), acting alone or combined (OWA), affected the fitness and physiological status of juvenile *D. cervinus*, using a multi-biomarker approach that combined whole organism (Fulton's condition index and specific growth rate), tissue (hepatosomatic and viscerosomatic indexes), cellular (erythrocytes and leukocytes percentages) and biochemical (i.e., superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase activities; total antioxidant capacity, lipid peroxidation, heat shock protein 70 and ubiquitin levels in fish muscle, liver, brain and gills) responses.

2. Materials and Methods

2.1. Husbandry and Acclimation

Juvenile *D. cervinus* were specifically targeted because (i) any physiological disruption at this earlier and more vulnerable life stage could affect the success of species recruitment and the subsequent maintenance of adult populations [15]; (ii) they inhabit tide pools and shallow coastal waters [16], thus being naturally exposed to wide ranges of abiotic conditions [16,17], but being vulnerable to climate change effects as they live close to their physiological thresholds and have a very strict thermal safety margin [14]; and (iii) their physiological responses to seawater warming and/or acidification at different levels of biological organization have not yet been determined.

Specimens with similar morphometric characteristics (weight = 5.7 ± 0.9 g; total length = 3.7 ± 0.4 cm (mean ± standard deviation, n = 48; for the purposes of this study, only 32 animals were used in total, i.e., the remaining fish were used in several analyses whose results are described in other works of the authors) reared until juvenile stage (proximate age of 3 months) at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal)) were transported in thermally isolated containers with constant aeration to Guia Marine Laboratory (MARE-FCUL, Cascais, Portugal), where the exposure trial took place. Upon arrival, the fish were randomly and equitably distributed in twelve rectangular incubating glass tanks (98 × 33 × 24.7 cm; each with 100 L of total volume; maximum animal density = 5 g body weight L^{-1}). Each incubation tank was equipped with an independent recirculating aquaculture system (RAS) containing biological (model FSBF 1500, TMC Iberia, Lisbon, Portugal) and physical filtration (protein skimmer; ReefSkimPro, TMC-Iberia, Lisbon, Portugal), as well as UV disinfection (Vecton 300, TMC Iberia, Lisbon, Portugal) and automatic temperature (Frimar, Fernando Ribeiro Lda, Barcarena, Portugal) and pH control (model Profilux 3.1 N, GHL, Bonn, Germany) via a solenoid valve system. In each tank, temperature and pH were controlled individually. Seawater pH levels were controlled using pH electrodes connected to a Profilux system apparatus, monitored every 2s and adjusted through the injection via air stones of either a certified CO_2 gas mixture (Air Liquide, Lisbon, Portugal) or CO_2-filtered air (using soda lime, Sigma-Aldrich, Lisbon, Portugal). Seawater used in the RAS was filtered (0.35 µm) and UV-sterilized (Vecton 600, TMC Iberia, Lisbon, Portugal). Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3) levels were checked every week by means of colorimetric test kits (Tropic Marin, Montague, MA, USA) and kept below 0.05 mg L^{-1}, 0.20 mg L^{-1} and 2.0 mg L^{-1}, respectively, through the daily removal of feces, as well as 20% seawater renewal in each incubation tank.

The fish were acclimated to laboratory conditions for 4 weeks before the beginning of the experiment, being kept under the following abiotic conditions: dissolved oxygen (DO) > 5 mg L^{-1}; temperature = 19 ± 0.4 °C; pH = 8.06 ± 0.10 units; salinity = 35.0 ± 1.0‰ using a WTW handheld Meter Multi 350i, Germany; and a photoperiod of 12 h light and 12 h dark (12 L:12 D). Seawater was partially replaced (around 20% of the total volume) daily, and the carbonate system speciation was calculated weekly from total alkalinity and pH measurements, allowing the determination of bicarbonate and pCO_2 values, following the methodology described by Sarazin et al. [18]. One week before initiating the seawater warming and acidification exposure scenarios, the temperature was slowly raised (1 °C per day) and the pH was slowly decreased (0.1 pH units per day), until reaching ΔT = +4 °C (i.e., 23 °C) and ΔpH = −0.3 units (i.e., 7.7 pH units; equivalent to ~1000 µatm pCO_2) in tanks simulating seawater warming and acidification conditions, respectively, following the IPCC projections (scenario SSP5-8.5; [2]).

2.2. Experimental Conditions

After the acclimation period, *D. cervinus* specimens were exposed to four treatments for 60 days, simulating either the average abiotic conditions of the present or the future temperature and pH conditions projected for 2100 [i.e., seawater warming (ΔT = +4 °C) and acidification (pCO_2~1000 µatm, equivalent to ΔpH = −0.3 pH units, [2])], with the following four treatments: (i) Control—seawater temperature set at 19 °C and pH at 8.0 (pCO_2~405 µatm) (according to current conditions used in juvenile zebra seabream rearing [19,20]; (ii) Ocean Warming (OW)—seawater temperature set at 23 °C and pH at 8.0; (iii) Ocean Acidification (OA)—seawater temperature set at 19 °C and pH at 7.7 (pCO_2~1000 µatm); and (iv) Ocean Warming + Acidification (OWA)—seawater temperature set at 23 °C and pH 7.7 (Supplementary Table S1).

Each treatment comprised three independent replicate tanks (n = 4 individualized animals per replicate/tank, i.e., 12 animals per treatment). During the experimental period, the fish were fed daily with a feed amount corresponding to ~3% of the individual average body weight (divided into two portions). The experimental feeds were produced by Sparos, Lda (Olhão, Portugal). Their proximate chemical composition and nutritional information are presented in Supplementary Table S2.

2.3. Sampling

After 60 days of exposure, 8 fish were randomly collected from each treatment (i.e., 2 to 3 fish collected from each replicate tank that composed each treatment) and euthanized with an overdose of tricaine methanesulfonate (MS-222) solution (2000 mg L^{-1} of MS-222, Sigma-Aldrich, St. Louis, MA, USA) buffered with sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, USA) in a ratio of 1:1 to reduce fish stress.

Weight (g) and total length (cm) were recorded for biometric data analysis. Immediately after euthanasia, a fraction of peripheral fish blood was collected with a syringe coated with 1 M EDTA (ethylenediaminetetraacetic acid, Honeywell, Riedel-de Haën, North Carolina, USA) by puncture of the caudal vein (n = 8 per treatment; n = 32 in total) to analyze the hematological parameters (see Section 2.6). Then, the fish were dissected, allowing the collection of muscle, liver, brain, gills and viscera (i.e., comprising the internal organs), being the first four tissues used in biochemical analyses (see Section 2.7). The liver and viscera were weighed (g) to evaluate animal fitness indexes (the hepatosomatic index—HSI and viscerosomatic index—VSI, respectively). Each fish tissue (muscle, liver, brain, gills) was individually homogenized using a T25 digital Ultra-Turrax device (Ika, Staufen, Germany) in ice-cold conditions using 2.0 mL of a phosphate-buffered saline solution (PBS; 140 mM NaCl, Panreac, Barcelona, Spain; 10 mM Na_2HPO_4, Sigma-Aldrich, USA; 3 mM KCl, Merck; 2 mM KH_2PO_4, Sigma-Aldrich, USA) at pH = 7.4 units. The crude homogenates were then centrifuged for 15 min at 10,000× g and 4 °C (VWR, model CT 15RE from Hitachi Koki Co., Ltd., Barcelona, Spain). The supernatant extracts were collected and immediately stored at −80 °C until further analyses.

2.4. Animal Fitness

The specific growth rate (SGR) was determined from the biometric data, according to the following formula:

$$\text{SGR}\ (\%\ \text{day}^{-1}) = \left(\frac{\ln(W_2) - \ln(W_1)}{t_2 - t_1}\right) \times 100 \quad (1)$$

where W_2 and W_1 are the animal weight (g) on day 60 and day 1, and t_2 and t_1 are the number of days between the first and last sampling day.

Fulton's condition index (K) was determined from the biometric data, according to the following formula [21]:

$$K = \left(W/L^3\right) \times 100 \quad (2)$$

where W is the animal weight (g) and L is the total length (cm).

The viscerosomatic index (VSI) and hepatosomatic index (HSI) were calculated, displaying the relationship between the fish weight and the respective organ weight, as follows [21,22]:

$$\text{VSI}(\%) = (\text{VW}/\text{W}) \times 100 \quad (3)$$

$$\text{HSI}(\%) = (\text{LW}/\text{W}) \times 100 \quad (4)$$

where VW is the fish viscera weight (g), LW is the fish liver weight (g) and W is the animal weight (g).

2.5. Hematological Parameters

Glass microscopy slides (VWR, Carnaxide, Portugal) were used to prepare fish blood smears (one slide per individual; n = 8 slides per treatment; n = 32 slides in total) that were stained following a method previously described by Kaplow and Ladd [23]. After being dried in air at room temperature for 60 s, the slides were fixed with 10% formaldehyde–ethanol (Honeywell, Riedel-de Haën, North Carolina, USA) and washed gently for 15 s in running tap water. Afterwards, the wet slides were incubated at room temperature for 30 s in a Coplin jar containing an incubation mixture [30% ethanol (Honeywell, Germany) + 0.3 g benzidine dihydrochloride (Honeywell, Riedel-de Haën, NC, USA) + 0.132 M $ZnSO_4.7H_2O$ (Merck, Darmstadt, Germany) + 1.0 g $NaC_2H_3O_2.3H_2O$ (CalbioChem, San Diego, CA, USA) + 3% H_2O_2 (Sigma-Aldrich, USA) + 1.0 N NaOH (Panreac, Barcelona, Spain) + 2.0 g safranin O (Fluka, Buchs, Germany)]. Subsequently, the slides were washed for 5–10 s in running tap water and dried in air. The microscope glass slides were mounted with dibutyl phthalate xylene (DPX; Scharlau, Barcelona, Spain) and observed through optical microscopy (OPTIKA Microscopes, Ponteranica, Italy). A minimum of 100 cells per fish were counted under the microscope (400× magnification). The total erythrocytes and leukocytes were counted, following the classification previously described in Gallo et al. [24], i.e., leukocytes = lymphocytes + macrophages.

2.6. Biochemical Analyses

All biochemical assays (n = 8 for each biomarker) were adapted to 96-well microplates (Greiner Bio-one, Kremsmünster, Austria) and performed in duplicate using chemicals of pro analysis grade or higher. The absorbances were measured using a microplate reader (BIO-RAD, Benchmark, Hercules, CA, USA).

The results were normalized and expressed in relation to the total protein content of the samples (mg of protein). The total protein content in the tissue extracts was measured using bovine serum albumin (BSA; NZYTech, Lisboa, Portugal) as a standard and assayed as described in Bradford [25].

Superoxide dismutase (SOD) activity (EC 1.15.1.1), based on enzymes' dismutation capability to convert superoxide radical into hydrogen peroxide, was carried out as described in Maulvault et al. [26], using nitroblue tetrazolium (NBT, Sigma-Aldrich, USA) and xanthine oxidase (XOD, Sigma-Aldrich, USA). The positive control used was SOD from bovine erythrocytes (Sigma-Aldrich, USA). Samples were read at 550 nm, and the results were expressed in percentage of NBT inhibition.

Catalase (CAT) activity (EC 1.11.1.6) was carried out as described in Maulvault et al. [26], where the reaction of the enzyme with methanol produces formaldehyde that is measured colorimetrically with Purpald at 540 nm. CAT activity was expressed as nmol of conjugate per minute per mg of protein. One unit was defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C.

Glutathione peroxidase (GPx) activity (EC 1.11.1.9) was determined according to Lawrence and Burk [27]. The co-substrate mixture was composed of sodium azide (Sigma-Aldrich, USA), nicotinamide adenine dinucleotide phosphate (NADPH, Sigma-Aldrich, USA), glutathione reductase (GSSG-reductase, Sigma-Aldrich, USA) and reduced glutathione (GSH, Sigma-Aldrich, USA). The reaction was initiated by the addition of hydroperoxide cumene (Sigma-Aldrich, USA), and the absorbance was read at 340 nm. The results were expressed in relation to the total protein content of the samples, as nmol per minute and per mg of protein.

Glutathione S-transferase (GST) activity (EC 2.5.1.18) was determined as described in Maulvault et al. [26], based on the conjugation of the thiol group of glutathione to the 1-chloro-2,4-dinitrobenzene (CDNB) substrate (Sigma-Aldrich, USA), causing an increase in the absorbance at 340 nm. The rate of increase in the absorption was directly proportional to the GST activity in the samples, which was expressed as μmol of conjugate per minute per mg of total protein.

The total antioxidant capacity (TAC) was determined according to the method described by Kambayashi et al. [28]. In this assay, the substrates used were myoglobin (Sigma-Aldrich, USA) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Alfa Aesar, Ward Hill, MA, USA). The reaction started with the addition of hydroxide peroxide (Sigma-Aldrich, USA), and the absorbance was read at 415 nm. The TAC was calculated from a six-point calibration curve, based on a series of Trolox standards ranging from 0.045 to 0.33 mM, and the results were expressed as µmol per mg of total protein.

Lipid peroxidation (LPO) was measured through the quantification of the specific end-product of the lipid oxidative degradation process, i.e., malondialdehyde (MDA), and assayed following the thiobarbituric acid reactive substances (TBARS) method as described by Ohkawa et al. [29]. The reagents added to the samples were sodium dodecyl sulfate (SDS 8.1%, Merck, Germany), trichloroacetic acid (TCA 20% pH = 3.5, Panreac, Spain) and thiobarbituric acid (TBA 1%, Sigma-Aldrich, USA). After centrifugation, supernatants were added to each well, and the absorbance was read at 530 nm. MDA concentrations were quantified based on a nine-point calibration curve ranging from 0.001 to 0.1 mM TBARS, performed with malondialdehyde bis-(dimethyl acetal) standards (MDA; Merck, Germany), being expressed as µmol per mg of total protein.

Heat shock protein 70 (HSP70/HSC70) was quantified using an indirect ELISA (Enzyme-Linked Immunoabsorbent Assay) as described in Maulvault et al. [26]. The primary antibody solution was anti-HSP70/HSC70 (Santa Cruz Biotechnology, Dallas, TX, USA; diluted to 1 µg mL^{-1} in 1% BSA solution;) and the secondary antibody solution was anti-mouse IgG Fc specific-alkaline phosphatase (Sigma-Aldrich, USA; diluted to 1 µg mL^{-1} in 1% BSA solution). After adding the substrate 4-nitrophenyl phosphate disodium salt hexahydrate (PnPP pH 8.5, Sigma-Aldrich, USA), the absorbance was read at 405 nm. The amount of HSP70/HSC70 in the samples was then calculated based on a nine-point calibration curve ranging from 0.0078 to 2 µg mL^{-1} of purified HSP70 active protein (OriGene Technologies, Rockville, MD, USA), being expressed as µg per mg of protein.

Ubiquitin (Ub) content was assessed by indirect ELISA as described in Pegado et al. [30]. Briefly, the primary antibody solution used was Ub (P4D1) Sc-8017, mouse monoclonal IgG (Santa Cruz Biotechnology, Dallas, TX, USA; diluted to 1.0 µg mL^{-1} in 1% BSA in PBS solution), and the secondary antibody solution was anti-mouse IgG Fc specific-alkaline phosphatase (Sigma-Aldrich, USA; diluted to 1 µg mL^{-1} in 1% BSA in PBS solution). After a washing step, an alkaline–phosphatase substrate composed of NaCl (Panreac, Spain), Tris-HCl (Sigma-Aldrich, USA), $MgCl_2$ (Sigma-Aldrich, USA) and PnPP (pH = 8.5, Sigma-Aldrich, USA) was added to each microplate well, and the absorbance was measured at 405 nm. The amount of Ub in the samples was then calculated based on a seven-point calibration curve ranging from 0.0125 to 0.8 µg mL^{-1} of purified Ub active protein (Santa Cruz Biotechnology, USA), being expressed as µg per mg of protein.

2.7. Statistical Analysis

Permutational univariate analyses of variance (PERMANOVAs) were performed for the tested variables (animal fitness indicators, hematological parameters and biochemical biomarkers) [31]. Euclidean distances were used to calculate the similarity matrix that was used to test whether the tested variables were affected by the treatments. Values of the pseudo-F statistic were run with partial sums of squares (Type III) and unrestricted permutation of raw data, and computed using 9999 permutations. Analyses were performed using PERMANOVA+ for PRIMER v6 (PRIMER-E Ltd., Plymouth, UK). As PERMANOVA is based on permutations, it is more robust to the assumptions of ANOVA [31]. Post hoc pair-wise comparisons were then performed using PERMANOVA to compare between treatments and variables. Differences were considered significant at $p < 0.05$.

To integrate all the results of biochemical biomarkers per tissue (i.e., muscle, liver, brain and gills) and animal fitness and hematological parameters, the integrated biomarker response (IBR) was calculated. The IBR, a simple multivariate graphic method—star plot—was calculated as previously described by Beliaeff and Burgeot [32] to allow a visual

integration of a set of early warning responses measured with biomarkers. The definition of the biological effect (inhibition or activation) of a given biomarker was based on its response to suboptimal abiotic conditions when compared to the control treatment conditions (i.e., seawater temperature = 19 °C and seawater pH = 8.0 units).

3. Results

3.1. Animal Fitness

Changes (%) in animal fitness indicators induced by ocean warming and/or acidification in relation to the average values obtained in the Control treatment are presented in Table 1. The specific growth rate (SGR), Fulton's condition index (K), viscerosomatic index (VSI) and hepatosomatic index (HSI) were significantly affected by the experimental treatment ($p < 0.05$; Table 2). SGR values significantly decreased after exposure to warming and/or acidification (i.e., −11% and $p = 0.005$ for OW, −42% and $p = 0.0007$ for OA, −49% and $p = 0.0007$ for OWA treatments; Figure 1). Regarding K values, only the combined effects of warming and acidification led to a significant decrease in K values (−35% in OWA, $p = 0.001$; Figure 1). Additionally, VSI values significantly decreased at warmer treatments (i.e., −52% in OW and −30% in OWA, $p = 0.001$; Figure 1), whereas HSI values significantly increased at acidified treatments (i.e., +10% in both OA and OWA, $p = 0.004$; Figure 1).

Table 1. Summary of changes (%) induced by acidification and/or warming in relation to average values obtained in Control treatment. "+" before the value indicates a significant increase, whereas "−" indicates a significant decrease ($p < 0.05$). Abbreviations: SGR—specific growth rate; K—Fulton's condition index; VSI—viscerosomatic index; HSI—hepatosomatic index; ERY—erythrocytes; LEU—leukocytes; SOD—superoxide dismutase; CAT—catalase; TAC—total antioxidant capacity; GPx—glutathione peroxidase; GST—glutathione S-transferase; LPO—lipid peroxidation; HSP70/HSC70—heat shock protein 70; Ub—ubiquitin; OW—simulated ocean warming, i.e., seawater temperature = 23 °C and pH 8.0 units; OA—simulated ocean acidification, i.e., seawater temperature = 19 °C and pH = 7.7 units; OWA—simulated ocean warming and acidification, i.e., seawater temperature = 23 °C and pH = 7.7 units; ns—no significant differences.

	SGR	K	VSI	HSI	ERY	LEU		SOD	CAT	TAC	GPx	GST	LPO	HSP70/ HSC70	Ub
OW	−11	ns	−52	ns	+1	−37	Muscle	+15	−62	−19	ns	−33	−45	+29	ns
							Liver	−30	+51	ns	−54	+51	−55	+98	−21
							Brain	+12	−14	ns	>+100	ns	−42	−88	−26
							Gills	ns	−42	+22	−40	+46	ns	+34	−33
OA	−42	ns	ns	+10	+1	−29	Muscle	ns	ns	ns	+60	−10	−34	ns	ns
							Liver	ns	+52	ns	−58	+53	ns	+68	ns
							Brain	−14	+16	+48	ns	ns	−29	−46	ns
							Gills	ns	−56	ns	−37	−6	ns	+83	−33
OWA	−49	−35	−30	+10	+1	−29	Muscle	+21	−73	−66	−12	−25	−60	−61	ns
							Liver	−25	+21	ns	−63	+83	−40	ns	ns
							Brain	−27	+9	+21	+24	ns	ns	−47	−21
							Gills	−30	−57	ns	−21	−18	ns	>+100	−40

Table 2. Summary of PERMANOVA permutation tests applied to assess the effects of the experimental treatments in the animal fitness indicators, hematological parameters and biochemical biomarkers of *Diplodus cervinus* juveniles. Significant differences ($p < 0.05$) are marked in bold. Abbreviations: SGR—specific growth rate, *K*—Fulton's condition index; VSI—viscerosomatic index; HSI—hepatosomatic index; SOD—superoxide dismutase; CAT—catalase; TAC—total antioxidant capacity; GPx—glutathione peroxidase; GST—glutathione S-transferase; LPO—lipid peroxidation; HSP70/HSC70—heat shock protein 70; Ub—ubiquitin.

	df	Pseudo-F	*p*-Value	Unique Perms
Fitness indicators				
SGR	3	60.68	0.0001	9949
K	3	8.65	0.0003	9956
VSI	3	97.93	0.0001	9954
HSI	3	5.03	0.0076	9947
Hematological parameters				
Erythrocytes	3	5.56	0.0133	9964
Leukocytes	3	5.56	0.0117	9954
Biochemical biomarkers				
Muscle				
SOD	3	8.20	0.0041	9938
CAT	3	30.93	0.0001	9921
TAC	3	181.71	0.0001	9904
GPx	3	33.99	0.0004	9922
GST	3	39.87	0.0001	9947
LPO	3	22.88	0.0001	9940
HSC70/HSP70	3	57.45	0.0001	9949
Ub	3	5.36	0.0066	9948
Liver				
SOD	3	26.12	0.0001	9929
CAT	3	79.80	0.0001	9943
TAC	3	21.95	0.0001	9932
GPx	3	46.63	0.0006	9925
GST	3	55.25	0.0001	9941
LPO	3	78.22	0.0001	9912
HSC70/HSP70	3	29.40	0.0001	9921
Ub	3	7.43	0.0044	9944
Brain				
SOD	3	35.96	0.0001	9900
CAT	3	13.08	0.0002	9934
TAC	3	25.41	0.0001	9945
GPx	3	102.87	0.0001	9940
GST	3	12.70	0.0018	9939
LPO	3	136.65	0.0001	9958
HSC70/HSP70	3	147.51	0.0001	9958
Ub	3	49.35	0.0003	9945
Gills				
SOD	3	38.34	0.0001	9944
CAT	3	124.39	0.0001	9913
TAC	3	19.75	0.0001	9925
GPx	3	144.25	0.0001	9890
GST	3	306.99	0.0001	9940
LPO	3	1.89	0.1895	9938
HSC70/HSP70	3	215.09	0.0001	9850
Ub	3	22.07	0.0011	9945

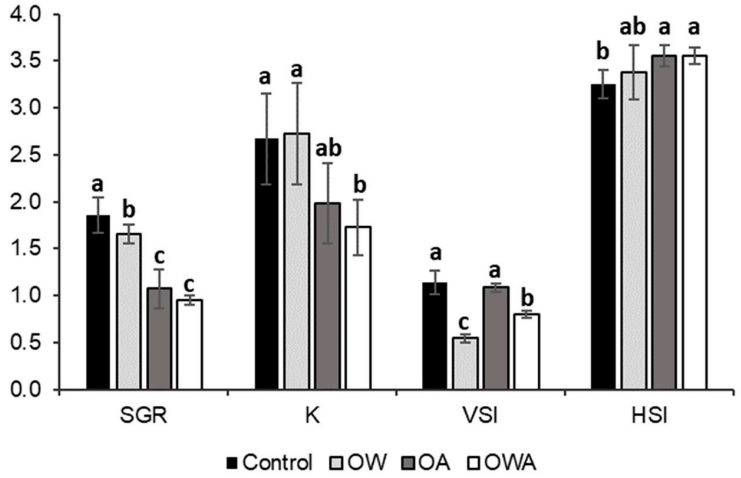

Figure 1. *D. cervinus* specific growth rate (SGR;% day^{-1}), Fulton's condition index (*K*), hepatosomatic index (HSI) and viscerosomatic index (VSI) after 60 days of exposure (mean ± standard deviation; $n = 8$). In each parameter, different letters denote significant differences between treatments ($p < 0.05$). Abbreviations: OW—simulated ocean warming, i.e., seawater temperature = 23 °C and pH 8.0 units; OA—simulated ocean acidification, i.e., seawater temperature = 19 °C and pH = 7.7 units; OWA—simulated ocean warming and acidification, i.e., seawater temperature = 23 °C and pH = 7.7 units.

3.2. Hematological Parameters

The changes (%) in fish hematological parameters induced by warming and/or acidification in relation to the average values obtained in the Control treatment are presented in Table 1. Erythrocyte and leucocyte percentages were significantly affected by the experimental treatment ($p = 0.01$; Table 2). The erythrocyte percentage was significantly higher upon fish exposure to all the abiotic stressors' treatments (i.e., +1% and $p = 0.008$ for OW, +1% and $p = 0.02$ for OA, +1% and $p = 0.03$ for OWA; Figure 2), while the leucocyte percentage presented an opposite trend (i.e., −37% and $p = 0.009$ for OW, −29% and $p = 0.02$ for OA, −29% and $p = 0.03$ for OWA; Figure 2).

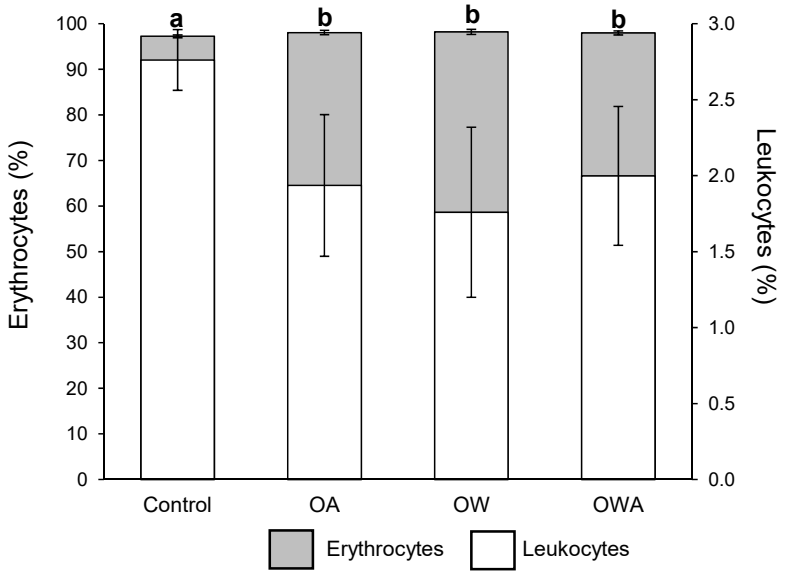

Figure 2. Percentage of erythrocytes and leukocytes (mean ± standard deviation; $n = 8$) in relation to total cell counts. Different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: OW—simulated ocean warming, i.e., seawater temperature = 23 °C and pH 8.0 units; OA—simulated ocean acidification, i.e., seawater temperature = 19 °C and pH = 7.7 units; OWA—simulated ocean warming and acidification, i.e., seawater temperature = 23 °C and pH = 7.7 units.

3.3. Biochemical Analyses

3.3.1. Antioxidant Defences and Lipid Peroxidation

The changes (%) in fish antioxidant defences and lipid peroxidation (LPO) induced by the abiotic stressors in relation to the average values obtained in the Control treatment are presented in Table 1. All the biomarkers were significantly affected by the experimental treatment in all tissues (muscle, liver, brain and gills, $p < 0.05$; Table 2), except for the LPO in fish gills ($p > 0.05$; Table 2).

In the muscle, whereas fish exposure to warmer treatments resulted in significantly higher superoxide dismutase (SOD) activity (i.e., +15% in OW and +21% in OWA, $p = 0.03$; Figure 3A), the opposite response pattern was observed in the catalase (CAT) activity and total antioxidant capacity (TAC) levels after fish exposure to these same treatments (i.e., CAT: −62% and −73%; TAC: −19% and −66%, in OW and OWA, respectively, $p = 0.03$; Figure 3B,C). Glutathione peroxidase (GPx) activity presented the opposite trend upon fish exposure to acidified treatments (i.e., +60% in OA and −12% in OWA, $p = 0.03$; Figure 3D). Furthermore, both glutathione S-transferase (GST) activity and LPO levels were significantly lower after exposure to warming and/or acidification (GST: −33%, −10% and −25%; LPO: −45%, −34% and −60%, in OW, OA and OWA, respectively, $p = 0.03$; Figure 3E,F).

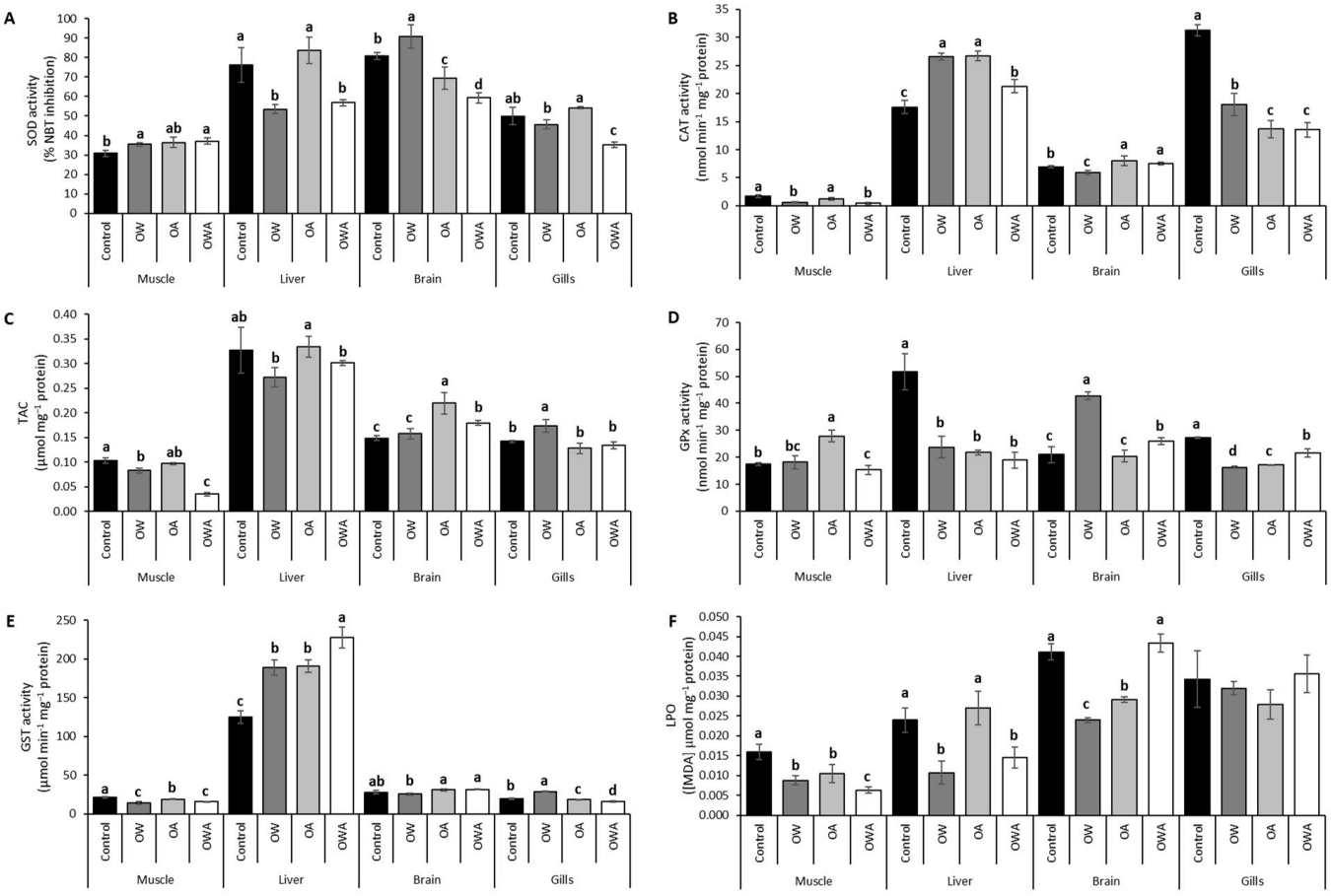

Figure 3. Antioxidant defences ((**A**)—SOD, % NBT inhibition; (**B**)—CAT, nmol min^{-1} mg^{-1} protein; (**C**)—TAC, µmol mg^{-1} protein; (**D**)—GPx, nmol min^{-1} mg^{-1} protein; (**E**)—GST, µmol min^{-1} mg^{-1} protein) and lipid peroxidation ((**F**)—LPO, [MDA] µmol mg^{-1} protein) in *D. cervinus* (muscle, liver, brain and gills; mean ± standard deviation; $n = 8$), after 60 days of trial. Different letters indicate significant differences ($p < 0.05$) between treatments, within the same tissue/biomarker.

In the liver, only fish exposure to warmer treatments resulted in significantly lower SOD activity (−30% in OW and −25% in OWA, $p = 0.03$; Figure 3A). Moreover, fish exposure to warming and/or acidification resulted in a different response pattern according

to the oxidative stress biomarker analyzed: (i) CAT and GST activities were significantly higher (CAT: +51%, +52% and +21%; GST: +51%, +53% and +83% in OW, OA and OWA, respectively, $p = 0.03$; Figure 3B,E); (ii) TAC levels were not altered (OW, OA and OWA, $p > 0.05$; Figure 3C), and (iii) GPx activity was significantly lower (−54%, −58% and −63% in OW, OA and OWA, respectively, $p = 0.03$; Figure 3D). Noteworthily, LPO levels were significantly lower at warmer treatments (−55% in OW and −40% in OWA, $p = 0.01$), but were not altered by acidification alone (OA, $p > 0.05$; Figure 3F).

In the brain, SOD and CAT activities presented the opposite trend upon fish exposure to all the abiotic stressors' treatments, i.e., warming alone induced significant higher SOD and lower CAT activities (SOD: + 12%; CAT: −14% in OW; $p = 0.03$), while acidified treatments induced significant lower SOD and higher CAT activities (SOD: −14% and −27%; CAT: +16% and +9%, in OA and OWA, respectively, $p = 0.03$; Figure 3A,B). As observed in the CAT activity response, TAC levels were significantly higher at acidified treatments (i.e., +48% in OA and +21% in OWA, $p = 0.03$; Figure 3C). Noteworthily, while GPx activity was significantly higher at warmer treatments (i.e., >+100% in OW and +24% in OWA, $p = 0.03$; Figure 3D), GST activity was not altered by any of the abiotic stressors (OW, OA and OWA, $p > 0.05$; Figure 3E). LPO levels were significantly lower after fish exposure to warming or acidification (−42% in OW and −29% in OA, $p = 0.01$), but did not change after fish exposure to their interactive effects (OWA, $p > 0.05$; Figure 4F).

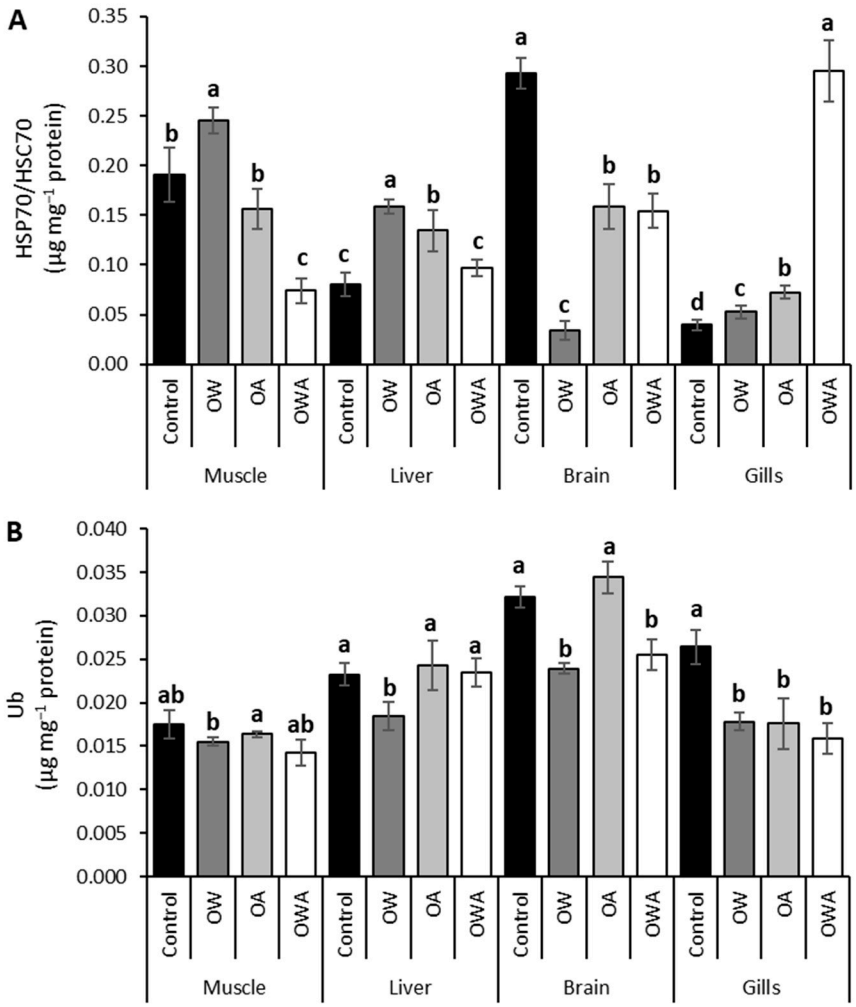

Figure 4. Heat shock protein 70 ((**A**)—HSP70/HSC70, µg mg^{-1} protein) and total ubiquitin ((**B**)—Ub, µg mg^{-1} protein) contents in *D. cervinus* (muscle, liver, brain and gills; mean ± standard deviation; $n = 8$) after 60 days of trial. Different letters indicate significant differences ($p < 0.05$) between treatments, within the same tissue/biomarker.

In the gills, SOD activity was only significantly lower after fish exposure to warming and acidification combined (−30% in OWA, $p = 0.03$; Figure 3A), whereas TAC levels were only significantly higher upon exposure to warming alone (+22% in OW, $p = 0.03$; Figure 3C). Noteworthily, while CAT and GPx activities were significantly lower upon fish exposure to all the abiotic stressors' treatments (CAT: −42%, −56% and −57%; GPx: −40%, −37% and −21% in OW, OA and OWA, respectively, $p = 0.03$; Figure 3B,D), LPO levels were not altered by any of the abiotic stressors (OW, OA and OWA, $p > 0.05$; Figure 3F).

3.3.2. Chaperoning and Protein Degradation

The changes (%) in heat shock protein 70 (HSP70/HSC70) and ubiquitin (Ub) contents induced by abiotic stressors in relation to the average values obtained in the Control treatment are presented in Table 1. All the biomarkers were significantly affected by the experimental treatment in each tissue (muscle, liver, brain and gills, $p < 0.05$; Table 2).

In the muscle, the HSP70/HSC70 concentration was only significantly altered at warmer treatments, presenting, however, an opposite trend (i.e., +29% in OW and −61% in OWA, $p = 0.03$; Figure 4A). Noteworthily, the Ub concentration was not altered by any of the abiotic stressors (OW, OA and OWA, $p > 0.05$; Figure 4B).

In the liver, while the HSP70/HSC70 concentration was significantly higher after exposure to warming or acidification (i.e., +98% in OW and +68% in OA, $p = 0.03$), it was not altered by their combined effects (OWA, $p > 0.05$; Figure 4A). Additionally, the Ub concentration was altered by warming alone, presenting a significantly lower concentration (−21% in OW, $p = 0.03$), but was not altered by acidified treatments (OA and OWA, $p > 0.05$; Figure 4B).

In the brain, while the HSP70/HSC70 concentration was significantly lower upon fish exposure to all the abiotic stressors' treatments (−88% and $p = 0.03$ in OW, −46% and $p = 0.01$ in OA, −47% and $p = 0.03$ in OWA; Figure 4A), the Ub concentration was significantly lower at warmer treatments (i.e., −26% in OW and −21% in OWA, $p = 0.03$), and it was not altered by acidification alone (OA, $p > 0.05$, Figure 4B).

In the gills, the HSP70/HSC70 and Ub concentrations presented opposite trends after exposure to warming and/or acidification (i.e., HSP70/HSC70: +34%, +83% and >+100%; Ub: −33%, −33% and −40% in OW, OA and OWA, respectively, $p = 0.03$; Figure 4A,B).

3.4. Integrated Biomarker Response

IBR index values calculated in each treatment for the biomarkers analyzed in the whole organism (i.e., animal fitness and hematological parameters) and in different fish tissues (i.e., biochemical biomarkers per tissue) are presented in Figure 5 (star plots are available in Supplementary Figure S1). Different treatments resulted in distinct biomarker responses in the whole organism, muscle, liver, brain and gills of *D. cervinus* juveniles. The combined effects of warming and acidification, OWA, yielded the highest IBR index value in terms of the biomarkers assessed at the whole organism level (OWA, IBR = 17; with SGR and K being the most responsive biomarkers, i.e., highest scores—S; S = 3) and also in fish muscle (OWA, IBR = 27; with SOD, CAT and Ub being the most responsive biomarkers; S = 3), brain (OWA, IBR = 16, with SOD being the most responsive biomarker; S = 3) and gills (OWA, IBR = 20, with GST and Ub being the most responsive biomarkers; S = 3). Only in fish liver, warming alone, OW, yielded the highest IBR index value (OW, IBR = 24, with Ub being the most responsive biomarker; S = 3). Regarding the lowest IBR index value, it was obtained in the Control treatment for all the studied biological levels, i.e., in the whole organism (IBR = 2) and in fish muscle (IBR = 4), liver (IBR = 3), brain (IBR = 2) and gills (IBR = 3).

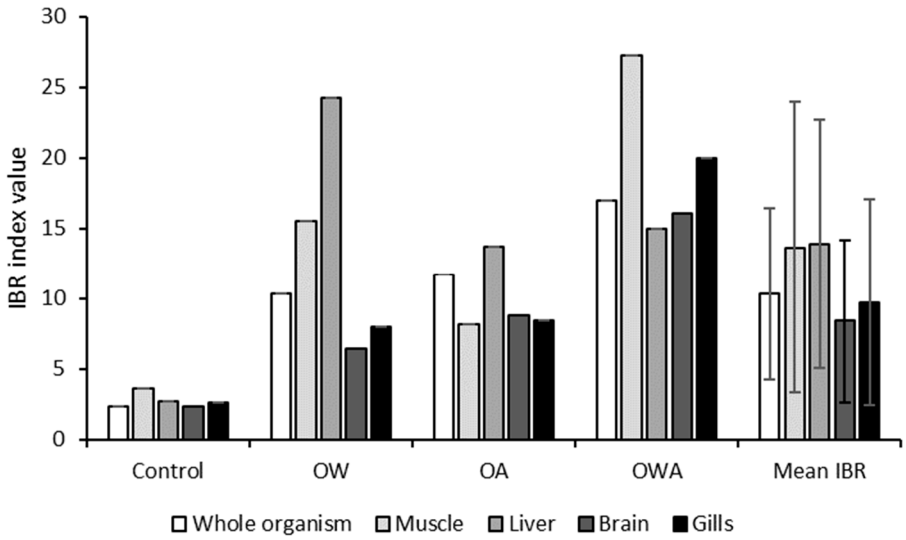

Figure 5. Total IBR index value and mean IBR (mean ± standard deviation; all treatments combined) for each treatment, including the different biomarkers analyzed in the whole organism and in the different fish tissues (muscle, liver, brain and gills).

4. Discussion

4.1. Animal Fitness

The results registered in the OW treatment (i.e., no changes in K and HSI, but a decreased VSI and SGR) suggest that the amount of feed provided to fish (which was the same in all treatments, ~3% of the individual average body weight) was sufficient to preserve animal welfare but was not enough to cope with the higher metabolic demands induced by a higher temperature and to enable animal growth. Similar results were observed in juveniles of other fish species after chronic exposure to warming: (i) white seabream (*Diplodus sargus*) for K and VSI after 56 days of exposure [33], and (ii) European seabass (*Dicentrarchus labrax*) and *D. sargus* for K and HSI after 28 days of exposure [34,35]. The absence of significant changes in K and VSI in fish exposure to OA indicates similar fish body and viscera conditions, whereas the increase in HSI indicates a higher accumulation of energy reserves or liver pathologies (e.g., [36,37]). Similar results were observed in (i) juvenile black seabream (*Acanthopagrus schlegelii*) for SGR and K after exposure to ΔpH = −0.3 units (pH = 7.8) for 56 days [38], and (ii) seabob shrimp (*Xiphopenaeus kroyeri*) for HSI after exposure to ΔpH = −0.7 units (pH = 7.3) for 5 days [39]. Fish exposure to OWA resulted in the accumulation of energy reserves or pathologies in the liver (increased HSI) and a reduced fish visceral condition associated with fat content (decreased VSI), which were associated with a reduction in both fish body condition (i.e., K) and SGR. This finding indicates that the interactive effects of ocean warming and acidification were more adverse than their individual effects. In previous studies, fish exposure to OWA has resulted in no significant changes in the studied fitness indicators (e.g., *Amphiprion melanopus* [40], *D. labrax* and *D. sargus* [34,35]). This different response pattern may be related to the fish species, life stage, degree of variation of the stress factors and/or the time of exposure. In our study, the results obtained in the OWA treatment suggest an antagonistic effect when compared with the OW and OA treatments, as it retrieved the same response observed in one or two treatments individually (SGR and HSI) or showed a reduction in the expected effect (VSI) [41,42]. It is known that long-term exposure to overeating and suboptimal water quality can prompt chronic stress that may trigger a delay in growth and consequent reduction in fish size [43]. Hence, the experimental conditions in this study may have resulted in chronic stress, observed in the parameters studied. The decrease found in SGR upon exposure to the abiotic stressors under study can affect *D. cervinus* juveniles' survival rate, which is a determinant for population fitness by defining the success of species recruitment and the subsequent maintenance of adult populations [15]. Moreover,

a reduced growth rate can affect the nutritional quality of wild and farmed fish species (e.g., [44,45]). In relation to aquaculture, lower fish growth directly impacts the revenues of farmers. Thus, in the future, the aquaculture for this species (and others in similar situations) will require an optimization of the quality and quantity of feed that is given to fish to overcome these impacts [46]. Yet the consequences can be even more severe in wild fish populations, where the amount of feed needed is not always available. Increased dietary needs have resulted in increased foraging behavior, which will ultimately lead to higher energy demands and increased mortality due to higher predation incidence [47].

4.2. Hematological Parameters

Blood cells contribute to metabolic processes and play a major role in species' immune responses. As so, blood parameters are used as indicators of fish physiological status and welfare [48]. Blood is a complex mixture of heterogeneous cell populations that includes erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes [48]. Most blood cells are erythrocytes, which ensure a sufficient supply of oxygen to the various tissues. Metabolic changes associated with stress responses increase tissues' oxygen requirements, so that greater numbers of erythrocytes are additionally recruited and mobilized from depots in the spleen [49]. However, the pattern of alteration in erythrocytes number is highly variable, depending on several factors (e.g., the type and magnitude of stressor, exposure time and fish species [48]). For instance, in this study, an increase in erythrocyte percentage was observed after exposure to warming and/or acidification. The same response pattern was observed in red spotted grouper (*Epinephelus akaara*) exposed to OW ($\Delta T = +6$ °C) for 6 weeks [50]. In contrast to our results, several researchers have reported no changes or a reduction in the number of erythrocytes after chronic exposure to heating and/or acidification in juveniles of different fish species (e.g., *Oreochromis mossambicus* [51], *D. labrax* [34] and *Scyliorhinus canicula* [30]).

Regarding leukocytes, a chronic exposure (60 days) to OW, OA and OWA reduced its number in juvenile *D. cervinus*. This response pattern is not surprising, given that chronic stress suppresses the immune response in fish, which consequently results in reduced leukocytes distribution and differentiation [43,52]. Corroborating our results, Islam et al. [53] observed a reduction in white blood cells number after *D. labrax* fingerlings' exposure to OW ($\Delta T = +8$ °C) for 30 days. The observed suppression in a fish's immune system upon exposure to warming and/or acidification will probably increase susceptibility to infection by pathogenic microorganisms [54], which will result in a higher need for the administration of antibiotics, pesticides and vaccines in aquaculture, and may lead to major mortalities in wild populations of zebra seabream [55,56]. Even though in aquaculture, probiotics and immunostimulants can be alternatives to antimicrobial agents [57], in wild fish populations, these alternatives are not available. Hence, climate change impacts are expected to be even more devastating in wild fish populations.

4.3. Antioxidant Defences and Lipid Peroxidation

The results of the present study indicate that a mismatch in SOD and CAT activities (i.e., the activity enhancement of the former corresponding to the activity inhibition of the latter and vice versa) was observed in fish muscle and liver at warmer temperatures (OW and OWA treatments) and in fish brain after exposure to warming and/or acidification. This response pattern suggests an ineffective detoxification of ROS that can potentially result in cell damage [58]. Similar stress responses have already been observed in the muscle of juvenile meagre (*Argyrosomus regius*) after exposure to similar experimental conditions (warming: $\Delta T = +5$ °C and acidification: ΔpCO_2 ~+1000 µatm, equivalent to $\Delta pH = -0.4$ units) for 28 days [59]. Additionally, the antioxidant response was not consistent among tissues and/or treatments. Several studies revealed that the cell's antioxidant defence depends on the stressor type and intensity, the tissue analysed and species (e.g., [34,59–62]). Corroborating our results in fish muscle, Lopes et al. [60] observed the same response patterns in the muscle of European glass eel (*Anguilla anguilla*) juveniles after

exposure to OW (i.e., decreased CAT activity and TAC levels, no changes in GPx activity; ΔT = +4 °C) and OWA (decreased TAC levels, CAT and GPx activities; ΔT = +4 °C and ΔpH = −0.4 units) for 12 weeks. Noteworthily, this response pattern was not the same in *A. anguilla* viscera [60], reinforcing the need for evaluating the antioxidant defence response in several tissues. Moreover, a decrease or absence of change in LPO levels in fish muscle (OA treatment), liver (OW, OA and OWA treatments), brain (OW and OWA treatments) and gills (OW treatment) was observed. This finding may be related to enhanced GPx or GST activities, whose function is to stop the LPO cascade reaction, thus reducing cellular damage from LPO by-products and their biomolecular adducts that take part in secondary deleterious reactions [63]. Hence, cell membrane phospholipids are protected, preventing damage to the membrane's structure and functioning [58,64]. A similar response pattern (i.e., enhanced GST activity with no changes in LPO levels) has already been observed in different juvenile marine fish species after exposure to similar experimental conditions for 28 days [34,35,59]. Moreover, de Fátima Pereira de Faria et al. [65] reported that although oxidative stress has been observed in juvenile pacu (*Piaractus mesopotamicus*) upon exposure to acute and chronic stressors, the LPO response varied according to the type of stressor. That is, increased LPO levels were observed after exposure to the acute stressor (fish were exposed to air for 3 min), while no changes in LPO levels were observed upon exposure to a chronic stressor (fish chased with a dip net for 5 min, twice a day, for 30 days) [65]. The results of the present study agree with this finding given that, although oxidative stress has been verified, no significant increases in LPO levels were observed after chronic exposure (i.e., 60 days) to warming and/or acidification.

In general, the results of our study agree with the ones observed by other researchers, i.e., the interactive effects of warming and acidification strongly affected the fish antioxidant machinery and that response was both biomarker- and tissue-specific (e.g., [34,35,59]). Overall, the results obtained in the OWA treatment suggest an antagonistic effect for most biomarkers analyzed in the different tissues, except for TAC and LPO in the muscle, GST in the liver and SOD in the gills, where OWA results suggest a synergistic effect [41,42].

The interaction of warming and acidification resulted in the inhibition of almost all antioxidant defences in the gills and muscle, except for TAC levels in gills and SOD activity in muscle. The same response pattern has been observed in the muscle of *A. anguilla* juveniles upon chronic exposure to similar experimental conditions [60]. This result may indicate that *D. cervinus* juveniles were exposed to stressful conditions (i.e., chronic exposure for 60 days to the combination of warming and acidification) and acclimation was not possible, resulting in the depression of the animal's metabolism (thus affecting protein synthesis), translating into an inhibition of antioxidant enzymes' activity [66]. However, in fish brain, the opposite response pattern was observed (enhanced CAT and GPx activities, increased TAC levels), while in the liver, antioxidant defences were both inhibited and enhanced (inhibition of SOD and GPx activities and enhanced CAT and GST activities). These results reinforce the statement that stressor response is tissue-specific.

4.4. Chaperoning and Protein Degradation

An increase in HSP70/HSC70 concentration was observed in the muscle (OW treatment), liver (OW and OA treatments) and gills (all treatments) that was accompanied by a decrease in or maintenance of Ub concentration, meaning that HSP production was sufficient to avoid irreversibly damaged proteins and the consequent activation of Ub. However, in fish brain, HSPs presented the opposite trend (i.e., a reduced HSP70/HSC70 concentration after exposure to the experimental treatments), decreasing or maintaining Ub concentrations. Previous studies have highlighted that the activation of chaperones is not a straightforward process, since the threshold for the induction/repression of protein synthesis can be influenced by several factors (e.g., stress levels, tissue, and species [34,57,60]). The decreased Ub concentration in several fish tissues (liver, brain and gills) can be explained by the lack of adequate energy for Ub synthesis under warming and/or acidification. As observed in several studies, Ub synthesis can be inhibited upon exposure to severe or

prolonged stress conditions, a consequence of physiological breakdown, given that protein synthesis is one of the most energy-demanding cellular processes (e.g., [35,60,67]). Matching the responses observed in fish antioxidant defences and lipid peroxidation, the results obtained in the OWA treatment suggest an antagonistic effect for most biomarkers analyzed in the different tissues except for (i) HSP70/HSC70 in fish gills, where OWA results suggest a synergistic effect [41,42], and (ii) HSP70/HSC70 in fish muscle, where the OWA treatment had an effect somewhat different from those observed when warming and acidification acted alone (i.e., OW enhanced the HSP70/HSC70 concentration, OA did not change it, while OWA diminished it).

In general, the results obtained in our study indicate that, in the OWA treatment, warming promoted a metabolic enhancement, whereas acidification inhibited it. This different pattern in the abiotic stressors' effects may be related to the degree of stress that this species can endure. That is, an increase of 4 °C is still within the thermal plasticity of this species, whereas, when it is combined with a decrease in 0.3 pH units, the degree of stress is already out of *D. cervinus*'s tolerance limits, leading to a metabolic reduction.

4.5. Integrated Biomarker Response (IBR)

The IBR analysis provides a broader overview of the fishes' physiological condition, allowing one to infer the degree of severity associated with exposure to different stress conditions. As observed in this study, biomarker response patterns varied with tissue, the type of stressor (i.e., warming vs. acidification) and number of stressors (i.e., individual vs. interactive effects). It was also observed that both the activation and inhibition of a given biomarker can reduce animal fitness. During oxidative stress, it is expected for an activation of antioxidant defenses to counteract the cytotoxic effects of ROS. However, their inactivation can also be observed when an organism cannot cope with severe and/or prolonged stress conditions.

In the muscle, brain, gills and whole organism, the highest IBR value occurred in the OWA treatment, indicating that in these tissues and at the organism level, the interactive effects of warming and acidification resulted in a worse health condition than their individual effects. However, in liver, the highest IBR index value was observed in the OW treatment. This result is related to the activation of chaperoning (HSP70/HSC70) mechanisms and the inhibition of Ub synthesis compared to the Control treatment, indicating a worse fish health condition. The indication of higher stress levels in fish exposed to warming alone or combined with acidification highlights the need for environmental monitoring and conservation strategies to prioritize geographic locations that are projected to be more affected by these climate change-related stressors in the future.

Nonetheless, it should be noted that the present findings are limited to the selected factors: (i) the severity degree of seawater temperature and pCO_2 levels; (ii) the marine fish species and life stage; (iii) the set of biomarkers; (iv) the origin of the biological model (i.e., aquaculture); and (v) the time of exposure.

5. Conclusions

The present findings show that exposure to the combination of warming and acidification has adverse effects on animal fitness (e.g., decreased SGR, *K* and VSI), hematological parameters (e.g., decreased leukocyte percentages) and biochemical biomarkers (e.g., the up-regulation and down-regulation of antioxidant defences and HSP70/HSC70) in *D. cervinus* juveniles. In general, the results suggest an antagonistic effect when warming and acidification are combined. Still, synergistic effects were observed in some biomarkers (e.g., TAC and LPO in muscle; GST in liver; SOD and HSP70/HSC70 in gills). The combination of warming and acidification yielded the highest IBR index value in the whole organism, muscle, brain and gills of *D. cervinus* juveniles, therefore suggesting that the effects of these stressors are more severe when they act together. The different responses observed after 60 days of exposure to the individual and interactive effects of warming and acidification in *D. cervinus* juveniles reinforce the need to develop studies where the

experimental design incorporates multi-stressor comparisons. The observed adverse effects at the different levels of biological organization (i.e., biochemical, cellular, tissue and the whole organism) indicate that warming and acidification, both individually and combined, can have a preponderant role on *D. cervinus* physiological status, which may likely defy the resilience of this species and, consequently, reshape its global circumtropical distribution and population dynamics. Future research on the effects of warming and acidification in marine fish species should also focus on transgenerational assessments, as such approaches are of the utmost importance to complement present outcomes and to acquire a deeper understanding regarding the long-term effects that may lead to the migration or extinction of a given marine species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/oceans5030033/s1, Figure S1: Star plots for the biomarkers analyzed in the whole organism (A) and in the different tissues: muscle (B), liver (C), brain (D) and gills (E) of *Diplodus cervinus* juveniles. Table S1: Seawater carbonate chemistry data (mean ± standard deviation): Abbreviations: CT—total carbon; pCO_2—carbon dioxide partial pressure; HCO_3^-—bicarbonate concentration; ΩAr—aragonite saturation state; ΩCa—calcite saturation state; pH_T—total scale; TA—total alkalinity; SW—seawater; Table S2: Formulation, ingredients and proximate chemical composition (% dry weight, dw) of fish feeds (mean ± standard deviation).

Author Contributions: Conceptualization, A.M., R.R. and P.A.; methodology, M.S.D., P.A. and A.L.M.; software, M.D.; validation, R.R. and P.A.; formal analysis, M.D., P.A. and A.L.M.; investigation, P.P.-F., M.S.D., A.M., R.R., P.A. and A.L.M.; resources, P.P.-F., M.S.D., A.M., R.R. and P.A.; data curation, M.D.; writing—original draft preparation, M.D.; writing—review and editing, M.D., P.P.-F., M.S.D., A.M., R.R., P.A. and A.L.M.; visualization, M.D., P.A. and A.L.M.; supervision, A.M., R.R. and P.A.; project administration, A.M., R.R. and P.A.; funding acquisition, A.M., R.R. and P.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project FISHBUDGET—Effects of climate change on marine fish energy budgets (PTDC/BIA-BMA/28630/2017) from the Portuguese Foundation for Science and Technology (FCT). The authors also acknowledge funding from FCT under the strategic project UIDB/04292/2020 granted to MARE and project LA/P/0069/2020 granted to the Associate Laboratory ARNET, and the strategic program of Applied Molecular Biosciences Unit—UCIBIO (UIDB/04378/2020). FCT also supported the contracts of P.A., A.M. and R.R. in the framework of the CEECIND 2017 (CEECIND/01739/2017/CP1387/CT0041), IF2014 (IF/00253/2014) and IF2013 programs (IF/01373/2013), respectively. The project Aqua-CLIMADAPT (PTDC/CTA-AMB/0592/2021) also supported the research contract of M.D.

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Faculty of Sciences of Lisbon University (n.°5/2019) and conducted according to national EU legal regulations (EU Directive 2010/63; Decree-Law n○ 113/2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: Dataset available on request from the authors.

Acknowledgments: The authors thank the Sparos Lda company for providing the fish feed, and the team from the EPPO-IPMA aquaculture pilot station (Olhão) for providing the juvenile fish for the trials.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Barange, M.; Bahri, T.; Beveridge, M.C.; Cochrane, K.L.; Funge-Smith, S.; Poulain, F. Impacts of climate change on fisheries and aquaculture. In *Synthesis of Current Knowledge, Adaptation and Mitigation Options*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2018; Volume 12, pp. 628–635. ISBN 978-92-5-130607-9.
2. IPCC. Summary for Policymakers. In *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*; Core Writing Team, Lee, H., Romero, J., Eds.; IPCC: Geneva, Switzerland, 2023; pp. 1–34. [CrossRef]

3. FAO. Fisheries and Aquaculture Information and Statistics Service. Available online: https://www.fao.org/fishery/en/information (accessed on 10 October 2023).
4. Stillman, J.H. Acclimation Capacity Underlies Susceptibility to Climate Change. *Science* **2003**, *301*, 65. [CrossRef] [PubMed]
5. Reynolds, W.W.; Casterlin, M.E. Behavioral Thermoregulation and the 'Final Preferendum' Paradigm. *Am. Zool.* **1979**, *19*, 211–224. [CrossRef]
6. Beitinger, T.L.; Bennett, W.A.; McCauley, R.W. Temperature Tolerances of North American Freshwater Fishes Exposed to Dynamic Changes in Temperature. *Environ. Biol. Fishes* **2000**, *58*, 237–275. [CrossRef]
7. Wendelaar Bonga, S.E. The stress response in fish. *Physiol. Rev.* **1997**, *77*, 591–625. [CrossRef] [PubMed]
8. Moore, M.N.; Depledge, M.H.; Readman, J.W.; Paul Leonard, D.R. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutat. Res.–Fundam. Mol. Mech. Mutagen.* **2004**, *552*, 247–268. [CrossRef] [PubMed]
9. IUCN. The IUCN Red List of Threatened Species. Version 2022-2. ISSN 2307-8235. Available online: https://www.iucnredlist.org (accessed on 10 October 2023).
10. Pajuelo, J.G.; Lorenzo, J.M.; Domínguez, R.; Ramos, A.; Gregoire, M. On the Population Ecology of the Zebra Seabream *Diplodus cervinus cervinus* (Lowe 1838) from the Coasts of the Canarian archipelago, North West Africa. *Environ. Biol. Fishes* **2003**, *67*, 407–416. [CrossRef]
11. Sbragaglia, V.; Nuñez, J.D.; Dominoni, D.; Coco, S.; Fanelli, E.; Azzurro, E.; Marini, S.; Nogueras, M.; Ponti, M.; Fernandez, J.D.R.; et al. Annual rhythms of temporal niche partitioning in the Sparidae family are correlated to different environmental variables. *Sci. Rep.* **2019**, *9*, 1708. [CrossRef] [PubMed]
12. Reef Life Survey. Diplodus cervinus. Available online: https://www.reeflifesurvey.com/species/diplodus-cervinus/ (accessed on 10 October 2023).
13. Bennett, W.A.; Judd, F.W. Comparison of Methods for Determining Low Temperature Tolerance: Experiments with Pinfish, *Lagodon rhomboides*. *Copeia* **1992**, *1992*, 1059–1065. [CrossRef]
14. Cereja, R. Critical thermal maxima in aquatic ectotherms. *Ecol. Indic.* **2020**, *119*, 106856. [CrossRef]
15. Di Franco, A. Mismatch in early life traits between settlers and recruits in a Mediterranean fish: Clue of the relevance of the settlement tail? *Acta Ichthyol. Piscat.* **2015**, *45*, 153–159. [CrossRef]
16. Dias, M.; Roma, J.; Fonseca, C.; Pinto, M.; Cabral, H.N.; Silva, A.; Vinagre, C. Intertidal pools as alternative nursery habitats for coastal fishes. *Mar. Biol. Res.* **2016**, *12*, 331–344. [CrossRef]
17. Madeira, D.; Narciso, L.; Cabral, H.N.; Vinagre, C. Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. *J. Sea Res.* **2012**, *70*, 32–41. [CrossRef]
18. Sarazin, G.; Michard, G.; Prevot, F. A rapid and accurate spectroscopic method for alkalinity measurements in sea water samples. *Water Res.* **1999**, *33*, 290–294. [CrossRef]
19. Coutinho, F.F. Dietary Protein Requirements and Intermediary Metabolism Response to Protein/Carbohydrate Ratio of Zebra Seabream (*Diplodus cervinus*, Lowe 1838) Juveniles. Master's Thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal, 2012; p. 48.
20. Ferreira, P.M.P. *Manual de Cultivo e Bioencapsulação da Cadeia Alimentar para a Larvicultura de Peixes Marinhos*; Instituto Nacional de Recursos Biológicos. IPIMAR: Santiago de Compostela, Spain, 2009; p. 235. ISBN 978-972-9372-37-7.
21. Ricker, W.E. Computation and interpretation of biological statistics of fish populations. *Fish. Res. Board Can. Bull.* **1975**, *191*, 1–382.
22. Goede, R.W. Organismic indices and an autopsy-based assessment as indicator of health and condition of fish. *Am. Fish Soc. Symp.* **1990**, *8*, 93–108.
23. Kaplow, L.S.; Ladd, C. Brief Report: Simplified Myeloperoxidase Stain Using Benzidine Dihydrochloride. *Blood* **1965**, *26*, 215–219. [CrossRef] [PubMed]
24. Gallo, S.S.M.; Ederli, N.B.; Bôa-Morte, M.O.; Oliveira, F.C.R. Hematological, morphological and morphometric characteristics of blood cells from rhea, Rhea Americana (Struthioniformes: Rheidae): A standard for Brazilian birds. *Braz. J. Biol.* **2015**, *75*, 953–962. [CrossRef]
25. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
26. Maulvault, A.L.; Barbosa, V.; Alves, R.; Custódio, A.; Anacleto, P.; Repolho, T.; Pousão-Ferreira, P.; Rosa, R.; Marques, A.; Diniz, M. Ecophysiological responses of juvenile seabass (*Dicentrarchus labrax*) exposed to increased temperature and dietary methylmercury. *Sci. Total Environ.* **2017**, *586*, 551–558. [CrossRef]
27. Lawrence, R.A.; Burk, R.F. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem. Biophys. Res. Commun.* **1976**, *71*, 952–958. [CrossRef]
28. Kambayashi, Y.; Binh, N.T.; Asakura, H.W.; Hibino, Y.; Hitomi, Y.; Nakamura, H.; Ogino, K. Efficient assay for total antioxidant capacity in human plasma using a 96-well microplate. *J. Clin. Biochem. Nutr.* **2009**, *44*, 46–51. [CrossRef]
29. Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351–358. [CrossRef]
30. Pegado, M.R.; Santos, C.P.; Pimentel, M.; Cyrne, R.; Sampaio, E.; Temporão, A.; Röckner, J.; Diniz, M.; Rosa, R. Lack of oxidative damage on temperate juvenile catsharks after a long-term ocean acidification exposure. *Mar. Biol.* **2020**, *167*, 1–10. [CrossRef]
31. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* **2001**, *26*, 32–46. [CrossRef]

32. Beliaeff, B.; Burgeot, T. Integrated biomarker response: A useful tool for ecological risk assessment. *Environ. Toxicol. Chem.* **2002**, *21*, 1316–1322. [CrossRef]
33. Anacleto, P.; Figueiredo, C.; Baptista, M.; Maulvault, A.L.; Camacho, C.; Pousão-Ferreira, P.; Valente, L.M.P.; Marques, A.; Rosa, R. Fish energy budget under ocean warming and flame retardant exposure. *Environ. Res.* **2018**, *164*, 186–196. [CrossRef] [PubMed]
34. Maulvault, A.L.; Barbosa, V.; Alves, R.; Anacleto, P.; Camacho, C.; Cunha, S.; Fernandes, J.O.; Pousão-Ferreira, P.; Rosa, R.; Marques, A.; et al. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. *Aquat. Toxicol.* **2018**, *202*, 65–79. [CrossRef] [PubMed]
35. Maulvault, A.L.; Camacho, C.; Barbosa, V.; Alves, R.; Anacleto, P.; Cunha, S.C.; Fernandes, J.O.; Pousão-Ferreira, P.; Paula, J.R.; Rosa, R.; et al. Bioaccumulation and ecotoxicological responses of juvenile white seabream (*Diplodus sargus*) exposed to triclosan, warming and acidification. *Environ. Pollut.* **2019**, *245*, 427–442. [CrossRef]
36. Chellappa, S.; Huntingford, F.A.; Strang, R.H.C.; Thomson, R.Y. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. *J. Fish Biol.* **1995**, *47*, 775–787. [CrossRef]
37. Santos, R.M.B.; Monteiro, S.M.V.; Cortes, R.M.V.; Pacheco, F.A.L.; Fernandes, L.F.S. Seasonal Differences in Water Pollution and Liver Histopathology of Iberian Barbel (*Luciobarbus bocagei*) and Douro Nase (*Pseudochondrostoma duriense*) in an Agricultural Watershed. *Water* **2022**, *14*, 444. [CrossRef]
38. Tegomo, F.A.; Zhong, Z.; Njomoue, A.P.; Okon, S.U.; Ullah, S.; Gray, N.A.; Chen, K.; Sun, Y.; Xiao, J.; Wang, L.; et al. Experimental Studies on the Impact of the Projected Ocean Acidification on Fish Survival, Health, Growth, and Meat Quality; Black Sea Bream (*Acanthopagrus schlegelii*), Physiological and Histological Studies. *Animals* **2021**, *11*, 3119. [CrossRef] [PubMed]
39. Augusto, A.; Ramaglia, A.C.; Mantoan, P.V. Effect of carbon dioxide-induced water acidification and seasonality on the physiology of the sea-bob shrimp *Xiphopenaeus kroyeri* (Decapoda, Penaeidae). *Crustaceana* **2018**, *91*, 947–960. [CrossRef]
40. Miller, G.M.; Kroon, F.J.; Metcalfe, S.; Munday, P.L. Temperature is the evil twin: Effects of increased temperature and ocean acidification on reproduction in a reef fish. *Ecol. Appl.* **2015**, *25*, 603–620. [CrossRef] [PubMed]
41. Crain, C.M.; Kroeker, K.; Halpern, B.S. Interactive and cumulative effects of multiple human stressors in marine systems. *Ecol. Lett.* **2008**, *11*, 1304–1315. [CrossRef] [PubMed]
42. Piggott, J.J.; Townsend, C.R.; Matthaei, C.D. Reconceptualizing synergism and antagonism among multiple stressors. *Ecol. Evol.* **2015**, *5*, 1538–1547. [CrossRef] [PubMed]
43. Nardocci, G.; Navarro, C.; Cortés, P.P.; Imarai, M.; Montoya, M.; Valenzuela, B.; Jara, P.; Acuña-Castillo, C.; Fernández, R. Neuroendocrine mechanisms for immune system regulation during stress in fish. *Fish Shellfish Immunol.* **2014**, *40*, 531–538. [CrossRef] [PubMed]
44. Prabu, E.; Felix, S.; Felix, N.; Ahilan, B.; Ruby, P. An overview on significance of fish nutrition in aquaculture industry. *Int. J. Fish. Aquat. Stud.* **2017**, *5*, 349–355.
45. Gao, X.; Zhai, H.; Peng, Z.; Yu, J.; Yan, L.; Wang, W.; Ren, T.; Han, Y. Comparison of nutritional quality, flesh quality, muscle cellularity, and expression of muscle growth-related genes between wild and recirculating aquaculture system (RAS)-farmed black rockfish (*Sebastes schlegelii*). *Aquac. Int.* **2023**, *31*, 2263–2280. [CrossRef]
46. Reid, G.K.; Gurney-Smith, H.J.; Flaherty, M.; Garber, A.F.; Forster, I.; Brewer-Dalton, K.; Knowler, D.; Marcogliese, D.J.; Chopin, T.; Moccia, R.D.; et al. Climate change and aquaculture: Considering adaptation potential. *Aquac. Environ. Interact.* **2019**, *11*, 603–624. [CrossRef]
47. Killen, S.S.; Marras, S.; McKenzie, D.J. Fuel, fasting, fear: Routine metabolic rate and food deprivation exert synergistic effects on risk-taking in individual juvenile European sea bass. *J. Anim. Ecol.* **2011**, *80*, 1024–1033. [CrossRef]
48. Seibel, H.; Baßmann, B.; Rebl, A. Blood Will Tell: What Hematological Analyses Can Reveal about Fish Welfare. *Front. Vet. Sci.* **2021**, *8*, 616955. [CrossRef] [PubMed]
49. Pearson, M.P.; Stevens, E.D. Size and hematological impact of the splenic erythrocyte reservoir in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **1991**, *9*, 39–50. [CrossRef] [PubMed]
50. Mofizur Rahman, M.; Bae Kim, H.; Ja Baek, H. Changes in Blood Cell Morphology and Number of Red Spotted Grouper, *Epinephelus akaara* in Response to Thermal Stress. *Dev. Reprod.* **2019**, *23*, 139–148. [CrossRef] [PubMed]
51. Kaya, H.; Hisar, O.; Yılmaz, S.; Gürkan, M.; Hisar, Ş.A. The effects of elevated carbon dioxide and temperature levels on tilapia (*Oreochromis mossambicus*): Respiratory enzymes, blood pH and hematological parameters. *Environ. Toxicol. Pharmacol.* **2016**, *44*, 114–119. [CrossRef] [PubMed]
52. Tort, L. Stress and immune modulation in fish. *Dev. Comp. Immunol.* **2011**, *35*, 1366–1375. [CrossRef]
53. Islam, M.J.; Slater, M.J.; Bögner, M.; Zeytin, S.; Kunzmann, A. Extreme ambient temperature effects in European seabass, *Dicentrarchus labrax*: Growth performance and hemato-biochemical parameters. *Aquaculture* **2020**, *522*, 735093. [CrossRef]
54. Mateus, A.P.; Power, D.M.; Canário, A.V.M. Stress and Disease in Fish. In *Fish Diseases*; Academic Press: Cambridge, MA, USA, 2017; pp. 187–220. [CrossRef]
55. Rodgers, C.J.; Furones, M.D. Antimicrobial agents in aquaculture: Practice, needs and issues. In *The Use of Veterinary Drugs and Vaccines in Mediterranean Aquaculture*; Options Mediterr.: Série A. Séminaires, Méditerranéens; n., 86; Rogers, C., Basurco, B., Eds.; CIHEAM: Zaragoza, Spain, 2009; pp. 41–59.
56. Magnadottir, B. Immunological Control of Fish Diseases. *Mar. Biotechnol.* **2010**, *12*, 361–379. [CrossRef] [PubMed]
57. Lieke, T.; Meinelt, T.; Hoseinifar, S.H.; Pan, B.; Straus, D.L.; Steinberg, C.E.W. Sustainable aquaculture requires environmental-friendly treatment strategies for fish diseases. *Rev. Aquac.* **2020**, *12*, 943–965. [CrossRef]

58. Halliwell, B.; Gutteridge, J.M.C. *Free Radicals in Biology and Medicine*; Oxford University Press: New York, NY, USA, 2015.
59. Maulvault, A.L.; Camacho, C.; Barbosa, V.; Alves, R.; Anacleto, P.; Pousão-Ferreira, P.; Rosa, R.; Marques, A.; Diniz, M.S. Living in a multi-stressors environment: An integrated biomarker approach to assess the ecotoxicological response of meagre (*Argyrosomus regius*) to venlafaxine, warming and acidification. *Environ. Res.* **2019**, *169*, 7–25. [CrossRef]
60. Lopes, A.R.; Figueiredo, C.; Sampaio, E.; Diniz, M.; Rosa, R.; Grilo, T.F. Impaired antioxidant defenses and DNA damage in the European glass eel (*Anguilla anguilla*) exposed to ocean warming and acidification. *Sci. Total Environ.* **2021**, *774*, 145499. [CrossRef]
61. Rodriguez-Dominguez, A.; Connell, S.D.; Leung, J.Y.S.; Nagelkerken, I. Adaptive responses of fishes to climate change: Feedback between physiology and behaviour *Sci. Total Environ.* **2019**, *692*, 1242–1249. [CrossRef] [PubMed]
62. Baldissera, M.D.; Souza, C.F.; Barroso, D.C.; Falk, R.B.; Wagner, R.; Baldisserotto, B.; Val, A.L. Disturbance of oxidant/antioxidant status and impairment on fillet fatty acid profiles in *Brycon amazonicus* subjected to acute heat stress. *Fish Physiol. Biochem.* **2020**, *46*, 1857–1866. [CrossRef] [PubMed]
63. Hayes, J.D.; McLellan, L.I. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **1999**, *31*, 273–300. [CrossRef] [PubMed]
64. Grim, J.M.; Hyndman, K.A.; Kriska, T.; Girotti, A.W.; Crockett, E.L. Relationship between oxidizable fatty acid content and level of antioxidant glutathione peroxidases in marine fish. *J. Exp. Biol.* **2011**, *214*, 3751–3759. [CrossRef] [PubMed]
65. de Fátima Pereira de Faria, C.; dos Reis Martinez, C.B.; Takahashi, L.S.; de Mello, M.M.M.; Martins, T.P.; Urbinati, E.C. Modulation of the innate immune response, antioxidant system and oxidative stress during acute and chronic stress in pacu (*Piaractus mesopotamicus*). *Fish Physiol. Biochem.* **2021**, *47*, 895–905. [CrossRef] [PubMed]
66. Sokolova, I.M. Energy-Limited Tolerance to Stress as a Conceptual Framework to Integrate the Effects of Multiple Stressors. *Integr. Comp. Biol.* **2013**, *53*, 597–608. [CrossRef]
67. Carter, C.G.; Houlihan, D.F. Protein synthesis. *Fish Physiol.* **2001**, *20*, 31–75. [CrossRef]