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Growth and Oxidative Stress of Clownfish *Amphiprion ocellaris* Reared at Different Salinities

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Abstract: Aquaculture of ornamental marine fish is often conducted in recirculating aquaculture systems (RAS) using artificial seawater. Considering the cost of salts to produce artificial seawater (salinity 35‰), we investigated the effect of different salinities (5, 15, 25, and 35‰) on survival, growth, and oxidative stress responses of juvenile clownfish *Amphiprion ocellaris*. All fish died when reared at salinity 5‰, but at all other salinities survival was ≥95% in the other treatment groups. There was no influence of salinity on growth and oxidative stress responses of clownfish reared at salinities 15, 25, and 35‰, except for the activity of glutathione S-transferase (GST) of fish reared at 25‰, which was significantly lower compared to those reared in salinity 35‰. The salinity of home aquariums is usually 35‰, so even though clownfish can be reared in brackish water, they need to be transferred to full strength seawater (35‰) in order to be commercialized. Therefore, we also evaluated the responses of acute transference of fish reared at 15 to salinity 35‰. There were no mortalities associated with acute salinity transference and no oxidative damage was observed either. The total capacity against peroxyl radicals (ACAP) was immediately increased after fish were placed in salinity 35‰, and remained high after 168 h (7 days), helping fish to deal with oxidative threats. In conclusion, it is possible to rear juvenile clownfish at 15‰ without harming growth or inducing oxidative stress, possibly reducing costs of water salinization. They can be transferred from brackish water to salinity 35‰ immediately before going to the retail market, with no mortality or oxidative damage.

Keywords: RAS; ornamental fish; artificial seawater

Key Contribution: Juvenile clownfish can be reared at salinity 15‰. Juvenile clownfish survive transference from salinity 15 to 35‰ without oxidative stress.

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1. Introduction

The marine aquarium industry is a millionaire business. Despite farmed fish playing an important role, it is still mostly based on wild-caught animals [1]. Approximately 11 million fishes are exploited from coral reef regions to supply the USA ornamental market alone [2], which accounts for 97.6% of the wild captured animals [3]. Several countries are involved in the aquarium industry, and both the collection and culture of marine animals play a growing role in their exports [4], which target not only home aquarists, but also large public aquaria [5]. The clownfish are one of the most representative groups of marine ornamental fish species. They belong to the subfamily Amphiprioninae, commonly named as anemonefishes. Both species, *Amphiprion percula* and *Amphiprion ocellaris*, are known as clown anemonefishes. The clownfish *A. ocellaris* is a widely distributed species in the ornamental industry, ranking fifth in the imports to the USA [2]. There are three different patterns of colors for *A. ocellaris*, of the variety "Black", endemic to the Darwin province

in Australia [6]. As a limited resource, it reaches high market values. Therefore, there is increasing concern with the status of the natural population [7]. In order to avoid a market that relies only on fisheries, it is important to stimulate its production in captivity, which can be a mechanism to help the conservation of the original stock.

The high cost of land and environmental issues make it difficult to establish business by the sea, and as such, there is an increasing tendency to move fish farms inland [8,9]. This is especially true of ornamental species, as due to the small biomass produced the demand for water exchange is not large. Anyway, production of marine fish inland relies on the use of artificial seawater or marine water that has to be transported to the farm site, and both strategies can be expensive. Recirculating aquaculture systems (RAS) are commonly used to produce ornamental fish. In addition to reducing the amount of water exchange, production of fish in RAS provides physical-chemical stability, and biosecurity [10]. Teleost fishes have the capacity to inhabit different environments, from fresh water to sea water, including brackish water. They deal with salinity changes using ionic and osmotic regulation of their body fluids, and they are considered euryhaline when they can withstand a large range of salinity, or stenohaline if they are strictly a fresh water or marine fish species [11].

The choice of euryhaline species for aquaculture is positive, because it increases the range of sites to implement a fish farm. Actually, production of fish in brackish water can give the benefit of rearing fish in a salinity close to their isosmotic point, which can save energy related to the ionic and osmoregulatory regulation, thus sparing energy for growth [12]. The energy sparing effect of intermediate salinity for growth was observed for juvenile yellow seabream *Acanthopagrus latus* and Asian seabass *Lates calcarifer* [13], and cobia *Rachycentron canadum* [14]. However, this is not necessarily true for all species as growth of shi drum *Umbrina cirrosa* reared at salinity 10 or 40‰ was not different [15]. Regarding Amphiprioninae, it has already been shown the successful use of brackish water on reproduction and larviculture of *Amphiprion akallopisos* [16] and *Amphiprion percula* [17]. However, considering the role of salinity on the oxidative status of Amphiprioninae species, studies have been limited to *Amphiprion melanopus*; there was an increased activity of enzymes involved on oxidative stress regulation after hypoosmotic acute challenge [18].

Oxidative stress can be defined as "an imbalance between prooxidants and antioxidants in favor of the former", leading to a disruption of redox signaling and control and/or molecular damage. The oxidative eustress responses occur when cells face low concentration of prooxidants, triggering adaptive response, while distress oxidative responses occur under a stressful situation caused by pathological or environmentally unsuitable changes [19,20]. Oxidative stress indexes can be used to understand the physiological alteration promoted by osmotic challenges [21–23]. Concerning oxidative indexes, glutathione S-transferase (GST) is a primary multifunctional enzyme able to detoxify and avoid damage influenced by salinity [24,25]. The total antioxidant capacity against peroxyl radicals (ACAP) measures the ability of the organism to combat ROS, which can cause oxidative damage [26]. The lipid peroxidation, indicated by the TBARS method, shows the lipid damage caused by ROS imbalance, which has also been related to salinity fluctuations [18,22].

The aim of the present study was to evaluate growth and oxidative status of clownfish reared with artificial water at different salinities while considering the economy to produce clownfish in brackish water. The consequences of abrupt transference of fish reared in brackish water (salinity 15‰) for full strength sea water (35‰) were also investigated.

2. Materials and Methods

2.1. Larval Stage and Ethics

The trials were conducted at the Laboratory of Marine Fish Culture (LAPEM), Institute of Oceanography, of the Federal University of Rio Grande (FURG), in Southern Brazil. Larvae of the clownfish *Amphiprion ocellaris* variety "Black" were reared at salinity 25‰ [27] with adaptations of previously described methodologies [28]. Larvae were reared in a tank with a static system. This tank was kept in a water table equipped with heat

controllers (26 ± 1 °C). The egg clutch obtained after a natural spawn was removed from the broodstock tank one day before hatching, nine days after they had been laid. After hatching, the larviculture was carried out using green water, and *Nannochloropsis oceanica* was added daily until 10 DPH (days post hatching). Larvae were fed on live prey, rotifers from 1 to 5 DPH, *Artemia* spp. nauplii from 4 to 10 DPH, and *Artemia* spp. metanauplii enriched with essential fatty acids from 8 to 15 DPH. Live prey density was kept between 2–5 prey/mL and the weaning process started at 12 DPH and finished at 20 DPH, when the fishes were fully weaned into dry commercial diets. Experiments were approved by the Ethics Committee on Animal Use (CEUA) of FURG (# 23116.000980/2014-52 and 23116.004798/2015-51).

2.2. General Methodology

2.2.1. Water Quality Analysis

The water quality was monitored every morning: for salinity (multiparameter 556 MPS, YSI, Yellow Springs, OH, USA); temperature and oxygen (Oxymeter 550A, YSI, Yellow Springs, OH, USA); pH (pH meter EcoSense pH 100A, YSI, Yellow Springs, OH, USA); alkalinity [29]; total ammonia nitrogen [30]; nitrite [31] and nitrate [32].

Ionic characterization of the water was made at the beginning and at the end of the trials. Flame photometry (Micronal B462, Piracicaba, Brazil) was used to measure sodium and potassium ions. Calcium and chloride were analyzed with commercial kits (Doles, Goiânia, Brazil), with protocol adaptations for reading in a microplate reader (Biotek, Synergy HT, Winooski, VT, USA). Water osmolality was determined with a pressure osmometer (Vapro 5600, Wercor Inc., Logan, UT, USA). Water samples were diluted in distilled water, as needed. All readings were made in triplicate samples.

2.2.2. Whole-Body Oxidative Status

Whole-body clownfish samples were homogenized (homogenizer Marconi, MA 590/Agata, Piracicaba, Brazil) in 1:4 proportion of sample weight and volume of an ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, pH 7.8, and 1% triton X-100 (*v*/*v*). After that, homogenized samples were centrifugated at 10,000× *g* at 4 °C for 30 min (SOLAB SL-703, Piracicaba, Brazil). Frozen supernatants were kept at −80 °C until they were used for the biochemical assays [33].

Total protein content of the supernatant was determined using a commercial kit (Proteínas Totais, Doles, Goiânia, Brazil) based on the biuret protein assay using bovine serum albumin (40 mg/mL) as standard. Samples were read using a 96 wells plate in a microplate reader (BioTek, LX 800, Winooski, VT, USA) at 550 nm.

The activity of glutathione-S-transferase (GST) was performed adding 1 mM of reduced glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) to 20 µL of sample homogenate. The resulting solution was read (absorbance 340 nm) every minute for 5 min (BioTek, LX 800, Winooski, VT, USA) [34].

The total antioxidant activity against peroxyl radicals (ACAP) was measured using the reactive oxygen species (ROS) protocol [26]. ABAP (20 µM 2,2-azobis-2-methylpropionamidine dihydrochloride), as peroxyl radical generator, or distilled water were added to 10 µL of sample homogenates containing 2.0 mg protein/mL using H_2DCF-DA (40 µM of 2′, 7′ dichlorofluorescein diacetate) as fluorochrome in the presence of free radicals generated by the incubation temperature (35 °C). Fluorescence values of the resulting fluorochromes were read every 5 min over 30 min (excitation 485 nm; emission 520 nm on Victor 2, Perkin Elmer, Shelton, CT, USA) Calculations were made using the following formula, where a lower relative area means a higher antioxidant capacity:

$$\text{ACAP} = (ROS_{\text{ with ABAP}} - ROS_{\text{ without ABAP}})/ROS_{\text{ without ABAP}}.$$

Lipid peroxidation (LPO) was assessed using the protocol for thiobarbituric acid reactive substances (TBARS) [35]. For that, 20 µL of homogenized sample was added to TBA (2-thiobarbituric acid), and the resulting fluorescence of the supernatant was read

(excitation 515 nm; emission 580 nm on Victor 2, Perkin Elmer, Shelton, CT, USA). TBARS levels were arranged into a 1,1,3,3-tetramethoxypropane (TMP) calibration curve.

2.3. Experiments

2.3.1. Trial 1: Effect of Salinity on Survival, Growth, and Oxidative Status of *A. ocellaris*

Juvenile clownfish (17.3 ± 0.4 mm, 88.3 ± 0.9 mg, 40 days old) produced at FURG hatchery were used for this experiment. They were 40 days old, and the stocking density was equal to 0.5 fish/L. Fish were fed ad libitum three times a day (9:00, 13:00, and 17:00 h) on a commercial diet (pellets 300–500 µm, Orange Grow, Inve, Salt Lake, UT, USA).

The experiment was conducted in four recirculating aquaculture systems (RAS). Each RAS was equipped with biological and mechanical filters, a protein skimmer, and a sump (60 L). Three rearing tanks (25 L) were attached to each RAS, made of fiber glass with black walls and white bottom. The trial was carried out in a room with a controlled photoperiod of 12 h day:12 h night.

The salinity of each system was equal to 5, 15, 25, and 35‰. Different salinities were obtained by diluting aquarium commercial salt (Red Sea, Houston, TX, USA) in dechlorinated tap water. Fish were acclimatized to the experimental salinities by lowering 5‰ per day until the desired salinities were achieved.

Growth of fish was followed by measuring and weighing them at the beginning of the experiment and after 30 and 60 days. Before measurements, fish were starved for 12 h and anaesthetized in a benzocaine hydrochloridrate bath (50 ppm). Performance of clownfish were assessed using the parameters described in the formula and statistics section (Section 2.4). At the end of the trial, all fish were measured and weighed. Five fish per tank were sampled and immediately euthanized in a benzocaine hydrochloride bath (300 ppm). Whole-fish were flash frozen in liquid nitrogen and stored at −80 °C until analysis of the oxidative status biomarkers described above.

Water quality was kept at appropriate levels for production of clownfish: dissolved oxygen was maintained above 6.2 mg O_2/L, and temperature was kept at 27.3 ± 0.0 °C. Total ammonia was below 0.1 mg N–NH_4 + NH_3/L, and nitrite and nitrate did not exceed 2 mg N–NO_2, or NO_3/L. Alkalinity and pH of brackish water were maintained at similar levels of seawater by adding sodium bicarbonate ($NaHCO_3$). Actual salinities, osmolarity, and ionic composition of the experimental water are shown in Table 1.

Table 1. Characterization of the water used for production of juvenile clownfish *Amphiprion ocellaris* in different salinities produced with artificial seawater.

Parameter	Salinity (‰)			
	5 *	**15**	**25**	**35**
Salinity (‰)	5.2 ± 0.07	15.3 ± 0.03	25.4 ± 0.05	35.3 ± 0.06
pH	8.1 ± 0.04	8.1 ± 0.01	8.1 ± 0.01	8.0 ± 0.01
Alkalinity (mg $CaCO_3$/L)	105.0 ± 5.0	152.0 ± 2.0	154.0 ± 2.0	153.0 ± 2.0
Osmolality (mOsm/kg)	128.8 ± 2.2	431.5 ± 13.5	764.5 ± 10.5	1060.5 ± 6.5
Na^+ (g/L)	3.5 ± 0.03	7.2 ± 0.05	12.3 ± 0.1	15.5 ± 0.06
Cl^- (g/L)	6.1 ± 0.02	8.2 ± 0.01	10.8 ± 0.03	12.7 ± 0.05
K^+ (mg/L)	109.2 ± 0.8	198.8 ± 0.0	388.1 ± 4.9	430.9 ± 0.0
Ca^{2+} (mg/L)	115.1 ± 0.8	220.5 ± 1.6	353.0 ± 2.1	447.8 ± 2.7

* Data of salinity 5‰ not included in the statistical analysis, because all fish died in this salinity during the first six days of the experiment. Mean ± standard error.

2.3.2. Trial 2: Response of *A. ocellaris* to Acute Transference from Brackish to Seawater

It was determined in trial 1 that fish reared at salinity 15‰ showed similar performance to those reared in seawater. Therefore, we wanted to evaluate the response of fish reared in brackish water after their abrupt transference to seawater. Then, juvenile clownfish (40 days old) were reared for 45 days at salinity 15‰ using the same procedures described above. The final fish weight was equal to 604.1 ± 27.1 mg and the total length measured

30.7 ± 0.5 mm. Six fish were sampled (time 0 h) and immediately 66 fish were divided into six groups of 11 fish and placed in six tanks (three tanks at salinity 15‰—group 15–15, and three tanks at salinity 35‰—group 15–35). Three fish from each tank were sampled at: 1, 24, and 168 h after they were transferred. The same procedures describe in trial 1 were used for fish euthanasia and storage of samples and the same oxidative status biomarkers were also used.

Water quality was kept at appropriate levels for production of clownfish: dissolved oxygen was maintained above 6.4 mg O_2/L, and the temperature was kept at 27.87 ± 0.07 °C. Total ammonia and nitrite were kept below 0.1 mg N–NH_4 + NH_3/L and 0.1 mg N–NO_2/L; nitrate did not exceed 20 mg N–NO_3/L. Alkalinity and pH of brackish water were maintained at similar levels of seawater by adding sodium bicarbonate ($NaHCO_3$). Actual salinities, osmolarity, and ionic composition of the experimental water are shown in Table 2.

Table 2. Characterization of the water used in the acute transference of juvenile clownfish *Amphiprion ocellaris* from brackish water to seawater (control 15–15‰, hyperosmotic shock 15–35‰).

Parameter	Salinity (‰)	
	15 (15–15)	**35 (15–35)**
Salinity (‰)	15.3 ± 0.07	35.2 ± 0.1
pH	8.2 ± 0.05	8.1 ± 0.04
Alkalinity (mg $CaCO_3$/L)	153.0 ± 3.0	157.5 ± 2.0
Osmolality (mOsm/kg)	447.7 ± 8.1	1036 ± 12.3
Na^+ (g/L)	7.8 ± 0.06	15.7 ± 0.2
Cl^- (g/L)	8.3 ± 0.02	12.3 ± 0.02
K^+ (mg/L)	198.5 ± 0.0	448.8 ± 5.1
Ca^{2+} (mg/L)	214.9 ± 0.9	434.9 ± 1.8

Mean ± standard error, data not submitted to statistical analysis.

2.4. Formulas and Statistics

2.4.1. Survival and Growth

Survival—S (%): initial number of fishes/final number fishes × 100;

Specific growth rate—SGR (%): (ln) final weight − (ln) initial weight/days of trial × 100;

Feed conversion rate—FCR (%): feed intake/weight gain × 100;

Weight gain—WG: final weight − initial weight;

Fulton's Condition Factor—K: final weight/final length^3 × 100.

2.4.2. Statiscal Analysis

The trial design was fully randomized. The Shapiro–Wilk's and Levene's tests were used to assure normality and homoscedasticity, respectively. If necessary, rank transformation was applied to meet the assumptions for analysis of variance (ANOVA) [36]. One-way ANOVA was used to observe differences among salinities on survival, weight, length, SGR, condition factor, weight gain, and feed conversion in trial 1. After rank transformations, two-way ANOVA was used in the second trial to observe differences after the acute transferences of fish, considering time and salinity; both were followed by the Newman–Keuls test to identify the differences. The minimum significance level was set at 5% ($p < 0.05$). All data were expressed as average ± standard error.

3. Results

3.1. Effect of Salinity on Survival, Growth, and Oxidative Status of Juvenile Clownfish

Twenty percent of the fish died two days after they were transferred to salinity 5‰. By day 4, mortality achieved 53.8%. All fish were dead six days after the experiment had begun. However, survival of clownfish reared at salinities 15, 25, and 35‰ were all above

95%, and no significant differences were found among them (p = 0.73). There were no significant differences for weight (p = 0.63) and length (p = 0.11) (Figure 1, Table 3), neither for any other parameters evaluated for fish reared at salinities 15, 25, and 35‰ (Table 3).

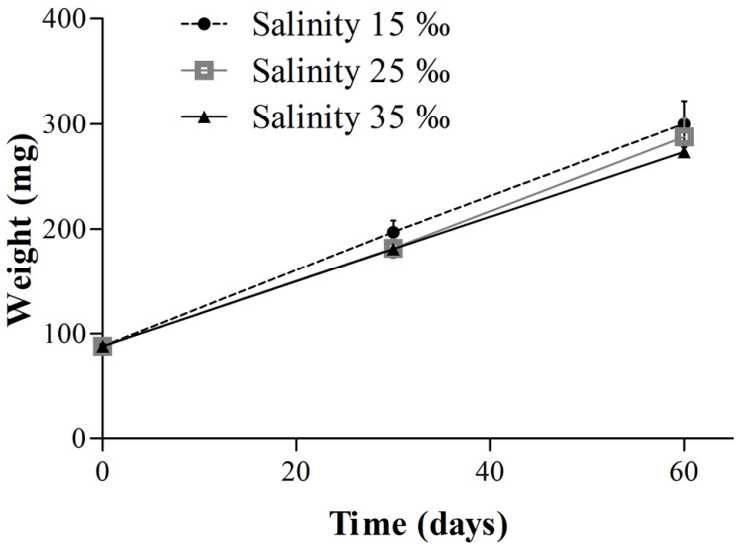

Figure 1. Weight of clownfish *Amphiprion ocellaris* reared in different salinities produced with artificial seawater. There were no significant differences (p = 0.11) for both.

Table 3. Survival, length, weight gain (WG), condition factor (K), feed conversion rate (FCR), and specific growth rate (SGR) (mean ± standard error) of juvenile clownfish *Amphiprion ocellaris* reared in different salinities produced with artificial seawater.

Parameter	Salinity (‰)			
	15	25	35	*p* Value
Survival (%)	95.0 ± 4.4	97.0 ± 4.4	95.0 ± 4.4	0.73
Length (mm)	23.8 ± 0.6	23.4 ± 0.5	23.2 ± 0.5	0.11
WG (mg)	211.1 ± 14.4	208.9 ± 20.6	177.7 ± 9.8	0.31
K	4.4 ± 0.2	4.6 ± 0.1	4.5 ± 0.1	0.55
FCR	1.9 ± 0.1	1.4 ± 0.1	1.5 ± 0.01	0.06
SGR (%/day)	2.0 ± 0.1	2.1 ± 0.3	1.7 ± 0.05	0.35

There were no significant differences ($p > 0.05$) for all parameters studied.

The protein content was 8% higher for fish reared at salinity 35‰ in comparison to those reared at lower salinities (p = 0.013). Regarding the other biochemical indexes, GST activity of fish reared at salinity 25‰ is 31% lower compared to fish reared at salinity 35‰ (p = 0.012), but there was no significant difference for GST activity between fish reared at 15 or 35‰ (p = 0.19). Meanwhile, there was no significant effect of salinity on ACAP (p = 0.73) and TBARS (p = 0.38) (Figure 2) after the 60 day rearing period.

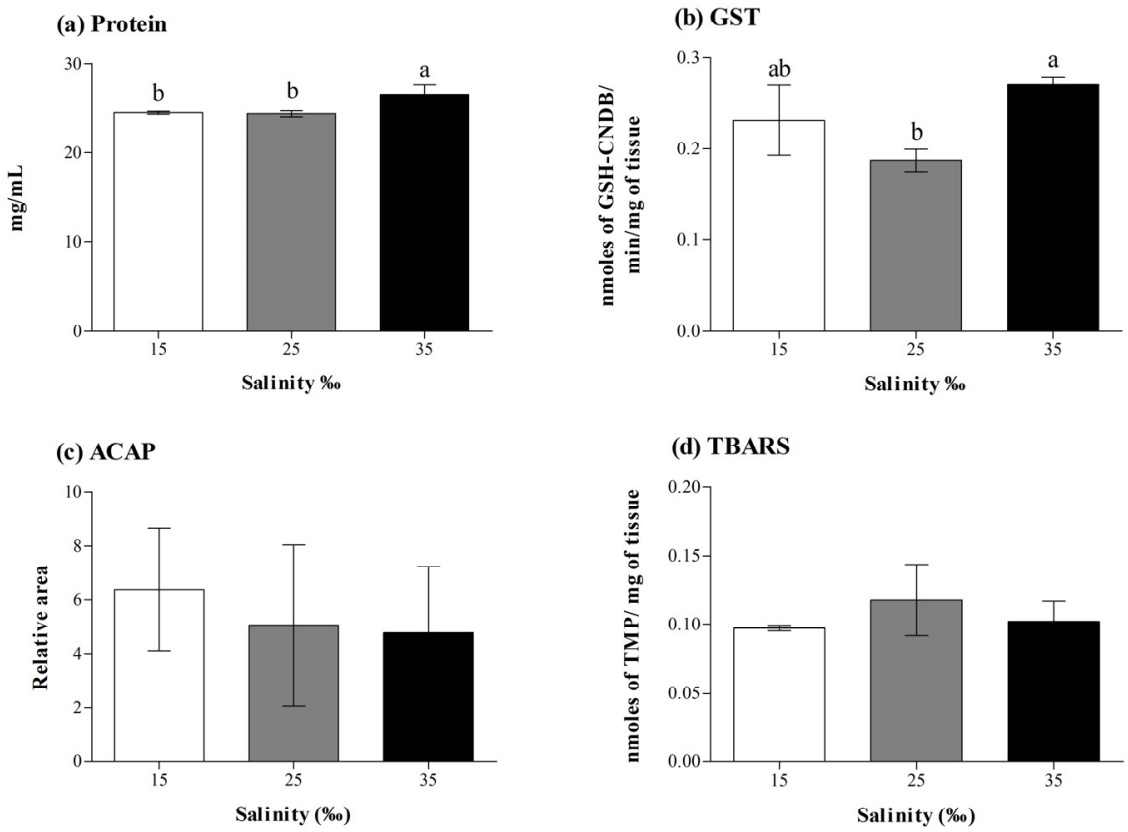

Figure 2. The effect of different salinities produced with artificial seawater on whole-body (**a**) protein content, (**b**) glutathione S-transferase activity (GST), (**c**) total antioxidant capacity against peroxyl radicals (ACAP), and (**d**) lipid peroxidation (TBARS) on juvenile clownfish *Amphiprion ocellaris*. Different letters indicate significant differences ($p < 0.05$).

3.2. Response of A. ocellaris to Acute Transference from Brackish Water to Seawater

All juvenile clownfish survived after transference from brackish water (15‰) to sea water (35‰), the same was true for the control group (transference from 15 to 15‰). No differences were observed for protein (p salinity = 0.30; p time = 0.26; p salinity × time = 0.33), GST activity (p salinity = 0.30; p time = 0.69; p salinity × time = 0.86), and TBARS (p salinity = 0.26; p time = 0.33; p salinity × time = 0.82) for fish in groups 15–15 and 15–35 at any trial times (Figure 2—protein; GST; TBARS). However, ACAP was increased in both groups, for those fish transferred from 15 to 15‰, ACAP content did not significantly increase, until 168 h after their transference, while ACAP for fish in the group 15–35 was higher within 1 h after they were transferred and remained elevated even at 168 h (p salinity < 0.01; p time = 0.02; p salinity × time = 0.21) (Figure 3).

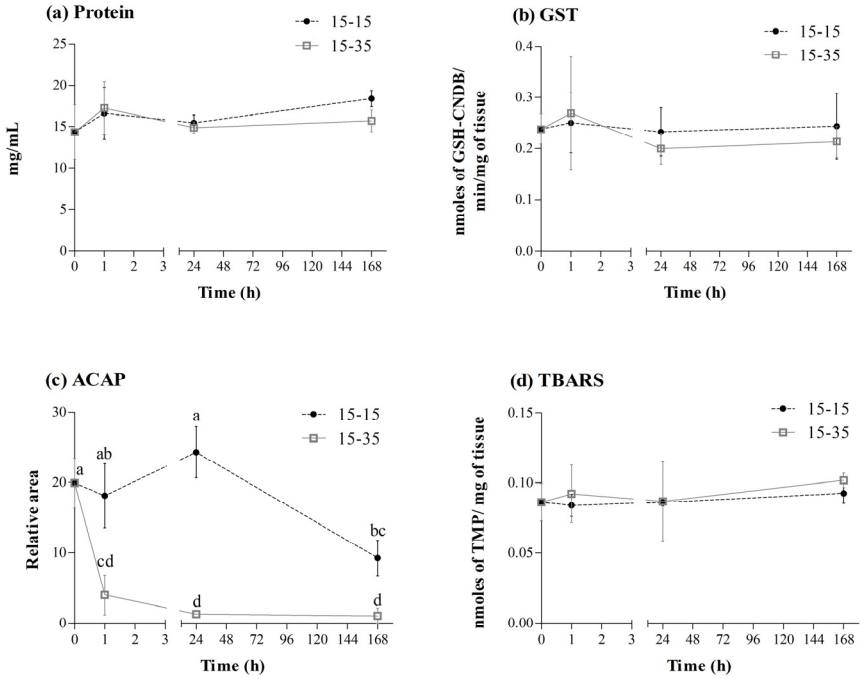

Figure 3. (**a**) Protein content, (**b**) glutathione S-transferase activity (GST), (**c**) total antioxidant capacity against peroxyl radicals (ACAP), and (**d**) lipid peroxidation (TBARS) after acute transference of juvenile clownfish *Amphiprion ocellaris* reared at salinity 15‰ to salinity 35‰; a control group, where fish were transferred from 15 to 15‰ was also analyzed. All data were transformed by rank. Different letters show differences between salinities across the time (two-way ANOVA, $p < 0.05$).

4. Discussion

The possibility to rear marine fish in different salinities is important, because production of euryhaline species inland, can reduce costs associated to land cost, environmental permits, and conflict of use in coastal areas [9]. This is especially true for the ornamental industry, because in general these fish farms operate at low stocking density, not demanding large volume of water exchange, and thus with reduced costs related to the lower amount of salt needed to provide a healthy environment for the fish. The stress responses associated with the salinity used for fish production are important, especially regarding survival and growth of the animals [37,38].

Amphiprioninae fish are typically marine species, and as such, they are expected to be stenohaline. However, juvenile *Amphiprion akallopisos* challenged with gradual salinity reduction survived all the way from full strength seawater down to salinity 6‰. The first mortality was observed when salinity reached 5‰, and total mortality of juvenile fish was observed only when water salinity dropped to 3‰ [16]. A similar result was observed in the present experiment; juvenile *A. ocellaris* acutely exposed to salinity 5‰ did not survive longer than 6 days. Regarding growth, some euryhaline fish have better performance at intermediated salinities (8–20‰) compared to those produced at full strength sea water [12]. This was proven to be true for seahorse *Hippocampus reidi* [39] and turbot *Scophthalmus maximus* [40]. However, this was not the case in the present study, as growth of juvenile clownfish was similar in the range of 15 to 35‰. Regarding the steno/eury-haline status of *A. ocellaris*, the results of survival and growth under different salinities suggest this is an euryhaline species, since it is unharmed when produced at salinity 15‰, and therefore does not require the use of full strength sea water for its commercial production. It is also note worth mentioning that larvae of *A. ocellaris* reared in brackish water show a lower percentage of asymmetric eyes, when compared to those reared in seawater, thus suggesting a less stressful condition at intermediate salinity [27].

The minimum commercial size of clownfish suggest by Hoff (1996) is equal to 25 mm [41]. Despite average growth not being affected by salinity within the range of 15–35‰, when

looking at the size distribution, a trend was observed for production of a larger percentage of fish approaching the commercial threshold size with intermediate salinities in this trial. The percentage of fish equal to or larger than 24 mm was equal to 40, 47, and 32%, respectively, with salinities 15, 25, and 35‰. Actually, within 60 days, it was only possible to observe fish larger than 30 mm at salinity 15‰. The possibility of producing more commercial sized fish faster suggests that production of clownfish in brackish water could be an advantage. Despite no detailed economic analysis being made, the possibility to rear clownfish at intermediate salinities also shows it is possible to reduce production costs, due to the reduce amount of salt needed for its production. Considering the same volume of water is needed to produce fish at salinities 15 and 35‰, the cost associated with the aquarium commercial salt is 57% smaller for the lower salinity. Lastly, production of fish in brackish water results in an effluent with lower salt content, which is important in reducing land and water salinization [42], especially if the fish farm is located inland, away from the coast.

The higher protein content of whole-body fish reared at the higher salinity level (35‰) can be related to the larger amount of proteins involved in the osmoregulation process; perhaps a more complex mechanism operates in seawater compared to fish reared in brackish water [11]. There was no impact on the oxidative status biomarkers analyzed (GST, TBARS, and ACAP) in clownfish reared for 60 days at salinity 15‰, corroborating the idea that clownfish do not need to be reared in seawater.

The growth experiment demonstrated clownfish coped well with chronic exposure to different salinities. Companies selling ornamental fish should be responsible for its well-being and survival for at least one week after it is sold [43]. Therefore, considering that water of home aquariums is maintained at salinity 30–35‰, it is necessary to ensure clownfish produced in brackish water are successfully transferred to sea water. The second challenge evaluated in the present study, the acute exposure from salinity 15 to 35‰, showed clownfish can also withstand the hyperosmotic shock. There were no mortalities related to this procedure, and the only alteration observed was the increased amount of antioxidant substances that made up the ACAP.

Apart from Salmonidae fish, which migrate from freshwater to the ocean, there is limited information regarding hyperosmotic shock in fish and its effect on oxidative status. Lipid damage was observed for the anadromous sturgeon, *Acipenser nacarii*, after an acute transference from freshwater to salinity 35‰. Alterations on the activity of enzymes related to oxidative status were also detected, especially regarding catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). which did not return to the initial values even after 20 days of exposure to 35‰ [22]. The antioxidant mechanism measured in the liver of yellow croacker (*Pseudosciaena crocea*) following a hyperosmotic shock (abrupt transference from salinity 26 to salinity 40‰) resulted in a reduction of lipid peroxidation in the first 6 h, but from there up to 48 h, a steady increase of lipid peroxidation was observed [44]. Regarding salinity stress on Amphiprioninae fish, alterations were observed on SOD, CAT, and GPx activity on *Amphiprion melanopus* transferred from salinity 35 to 17.5‰, as well as high levels of lipid peroxidation [18]. This suggests stress salinity can affect the oxidative status of fish, even when fish are exposed to salinities faced by these species in nature. On the other hand, the hyperosmotic salinity challenge of the present study did not cause effects on GST or TBARS, but an almost immediate and sustained increase of ACAP.

The role of ACAP in the oxidative status of fish exposed to hyperosmotic shock has not been studied. However, the increased amount of antioxidant reserves observed for fish reared at salinity 15 and transferred to salinity 35, shows the importance of understanding the role of ACAP in the oxidative status of fish after salinity challenges, especially because the increased antioxidant capacity helped to prevent lipid peroxidation [26,45]. The overall output of the hyperosmotic challenge shows that clownfish produced in brackish water can be transferred to seawater without being harmed.

5. Conclusions

The juvenile clownfish *A. ocellaris* does not survive in salinity 5‰. Nevertheless, there is no influence of salinity on growth and survival in the 15 to 35‰ salinity range. Fish reared at salinity 15‰ do not have alterations in their oxidative status and lipid peroxidation compared to those reared in sea water. It is also important to mention that there is no lipid peroxidation caused by acute transference of fish from 15 to 35‰, and most important, there is no mortality associated with the hyperosmotic challenge. This is a decisive feature for the aquarium industry, since fish can be safely transferred from the production site and placed in a seawater aquarium.

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