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Physiological parameters of Brazilian silverside, *Atherinella brasiliensis*, embryos exposed to different salinities

Carolina Brioschi Delpupo[1], Chris I. Espeland[2], Aline Karl Araújo[3], Jackson de Souza-Menezes[4], Daniela M. Pampanin[2] and Natália Martins Feitosa[1]

Correspondence:
Natália Martins Feitosa
nataliafeitosa@gmail.com

Information regarding organism changes due to the variation of abiotic factors such as salinity are essential in both ecotoxicological and environmental monitoring studies. For this reason, the Brazilian silverside (*Atherinella brasiliensis*) embryos were exposed to different salinity conditions (10–35) for 12 days and changes at molecular and individual levels were assessed. The embryos did not present alterations in the morphology or hatching during their development. However, they showed an increase in heart rate after seven days, close to the hatching period. The expression of the cystic fibrosis transmembrane regulator (*cftr*), one of the channels responsible for osmoregulation, was cloned and it was not significantly affected by the exposure. The obtained results indicated that the Brazilian silverside embryos acclimate in a broad range of salinities and can be used to study fish response at environmentally relevant conditions. In addition, this species can be used to assess the risk related to chemical compounds which toxicity may vary in different salinity conditions.

Keywords: Salinity tolerance, Silverside, *cftr*, Embryo test, Heart rate.

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1 Laboratório Integrado de Biociências Translacionais (LIBT), Instituto de Biodiversidade e Sustentabilidade (NUPEM), Universidade Federal do Rio de Janeiro, Av. São José do Barreto, 764, 27965-045 Macaé, RJ, Brazil. (CBD) caroldelpupo98@gmail.com ORCID, (NMF) nataliafeitosa@gmail.com (corresponding author).

2 University of Stavanger, Faculty of Science and Technology, Department of Chemistry, Bioscience and Environmental Engineering, N-4036 Stavanger, Norway. (CIE) ci.espeland@gmail.com, (DMP) daniela.m.pampanin@uis.no.

3 Unidade Integrada de Imagem, Instituto de Biodiversidade e Sustentabilidade (NUPEM), Universidade Federal do Rio de Janeiro, Av. São José do Barreto, 764, 27965-045 Macaé, RJ, Brazil. (AKA) alinekarl.ufrj@gmail.com.

4 Laboratório Integrado de Ciências Morfofuncionais (LICM), Instituto de Biodiversidade e Sustentabilidade (NUPEM), Universidade Federal do Rio de Janeiro, Av. São José do Barreto, 764, 27965-045 Macaé, RJ, Brazil. (JSM) jacksonmenezes@gmail.com.

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As informações relativas a mudanças nos organismos causadas por variações abióticas, como a salinidade, são essenciais para estudos de ecotoxicologia e monitoramento ambiental. Por esta razão, embriões da espécie eurialina do peixe-rei, *Atherinella brasiliensis*, foram expostos a diferentes condições de salinidade (10–35) por 12 dias para analisar possíveis mudanças morfológicas e moleculares. Os embriões não apresentaram alterações fenotípicas ou de eclosão durante o seu desenvolvimento. No entanto, eles demonstraram aumento no ritmo cardíaco após sete dias, próximo ao período de eclosão. A expressão do regulador transmembranar da fibrose cística (*cftr)*, um dos canais responsáveis pela osmorregulação, foi clonado e analisado, mas não apresentou variação significativa. Os resultados obtidos indicaram que os embriões de peixe-rei podem se aclimatar a uma ampla faixa de salinidades e podem ser usados para estudar a resposta dos peixes a condições ambientalmente relevantes. Adicionalmente, esta espécie pode ser usada para a avaliação de risco relacionada a compostos químicos, cuja toxicidade pode variar em diferentes condições de salinidade.

Palavras-chave: Tolerância salinidade, *cftr*, Teste de embrião, Frequência cardíaca, Peixe-rei.

INTRODUCTION

The South American Atlantic coast consists of several different types of marine and estuarine areas, where no resident species have been adapted for use in the laboratory. The Brazilian silverside *Atherinella brasiliensis* (Quoy & Gaimard, 1825) is a small pelagic fish, reaching up to about 16 cm in length, that resides in estuarine areas from Venezuela to South-Eastern Brazil (Fernandez *et al*., 2011; Souza-Bastos, Freire, 2011; Pichler *et al*., 2015). It is an ecologically relevant specie as it plays an important part in the trophic food chain, as food for birds and commercial fish species (Menezes *et al*., 2003). Its diet is opportunistic and varies from microalgae, copepods, amphipods and crustaceans to smaller fish (Chaves, Vendel, 2008; Rocha *et al*., 2008; Contente *et al*., 2010). For those reasons, the Brazilian silverside embryo is considered an appropriate candidate model organism for environmental risk assessments (Feitosa *et al*., 2021). A previous study from our research group demonstrated that embryos of the Brazilian silverside hatch and survive in a salinity range between 10 and 35 and only high temperatures (*e.g*., 28 °C) affected their survival rates (Feitosa *et al*., 2021). However, some morphological characteristics of the eggshell, egg lay frequency and physiological aspects under different salt conditions remained to be investigated. Therefore, aspects of egg laying by adult fish in the laboratory and chorion morphology were analyzed in this study.

Teleost fish rely on chloride secretory activity cells, named ionocytes or mitochondrion-rich cells, with several transmembrane protein channels, as osmoregulation mechanisms (Bodinier *et al*., 2009; Fridman, 2020). The osmoregulation of teleost fish involves different tissues including mainly: the integument, the gills, the digestive tract, and the kidney. The participation of each tissue depends on the ontogeny stage of the individual. In the first stages of life, the integument is the main responsible for this mechanism, later

with the development of the gills, they assume this function (Hiroi *et al*., 2005; Bodinier *et al*., 2009). Fish osmoregulation occurs in response to the external salinity, which rapidly upregulates or downregulates salt ions flow when the medium changes (Marshall, Singer, 2002; Bodinier *et al*., 2009). The main ion transport proteins responsible for the osmotic regulation are Na+/K+-ATPase, Na+/K+/2Cl– cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR) (McCormick *et al*., 2003; Hiroi *et al*., 2005). The ionocytes are classified as I, II, III and IV type cells depending on the presence and organization of those ion transporters. CFTR proteins are present only in the type IV ionocytes and its presence changes when tilapia embryos are transferred from freshwater to saline water or *vice versa* (Hiroi *et al*., 2005).

The protein cystic fibrosis transmembrane conductance regulator (CFTR) belongs to a superfamily of ATP-binding cassette (ABC) transport. It is the only ion channel of this superfamily, which main function is the osmoregulation. Its activity is regulated by the cyclic AMP/protein kinase A (PKA)-dependent phosphorylation (Zhang, Chen, 2016) and it is located in the apical area of cells, which assure its involvement in the chloride secretion by marine fish (Marshall, Singer, 2002). The CFTR has been identified in many species, from bacteria to human (Zhang, Chen, 2016), including several fish, such as *Fundulus heteroclitus*, *Takifugu rubripes*, and *Salmo salar* (Bodinier *et al*., 2009; Lema *et al*., 2018). In humans, mutations in this gene cause cystic fibrosis, a lethal disease (Marshall, Singer, 2002; Bodinier *et al*., 2009). In zebrafish development, Cftr is important for the Kupffer´s vesicle expansion, affecting laterality of the organs and gut development (Bagnat *et al*., 2013; Roxo-Rosa *et al*., 2015). In fish, changes in *cftr* expression can indicate an osmotic stress (Singer *et al*., 1998; McCormick *et al*., 2003; Scott *et al*., 2004; Lema *et al*., 2018).

The aim of the present study was to assess the response of the Brazilian silverside embryos to salinity changes, which may support the suitability of this species for ecotoxicological studies. Since the toxicity of various environmental contaminants may vary depending on abiotic factors such as salinity (*e.g*., metals) (Bielmyer *et al*., 2012), it is important to determine these responses and ensure that the species is suitable for environmentally relevant research.

MATERIALS AND METHODS

Fish maintenance. Between 15–20 adult fish in 1:1 ratio of females:males were maintained in an 130 L aquarium at 24 ± 1°C, containing activated carbon filters with a 14h/10h light/dark cycle. Fish were kept as described in Feitosa *et al*. (2021), at 20 ± 1 salinity with artificial sea water (Red Sea salt and Instant Ocean® Sea Salt), 6.5 < pH < 7.3 and were fed three times a day with Sera Vipan, Sera Vipagran and Alcon Basic®. The embryos were obtained from adults maintained in laboratory according to Feitosa *et al*. (2021). Voucher specimens are deposited in the Instituto de Biodiversidade e Sustentabilidade (NUPEM), Macaé: NPM 6185.

Embryo medium preparation. Artificial seawater at various salinities (*i.e*., 10, 15, 20, 25, 30, and 35) was prepared by dissolving Instant Ocean Sea Salt in distilled water and checked with a refractometer (Feitosa *et al*., 2021). Embryo medium solutions were stored in 50 ml polystyrene falcon tubes in a dark incubator at 25 ± 1 °C.

Embryo preparation. The fish maintained in the laboratory conditions were able to lay eggs at a constant rate, demonstrating a certain pattern. This was an important observation, which helped programming experiments with embryos along the year.

Eggs were released in the water, and collected by hand (Feitosa *et al*., 2021). Fertilized eggs were separated and cleaned, as described in Feitosa *et al*., 2021, and from less than 9 h post fertilization (< 9 hpf) embryos were observed daily. Embryos were subsequently washed three times in filtered seawater from the fish tank and distributed in a 24-well plate.

In the static experiment, with no solution change, 10 eggs were used for each salinity treatment, one egg per well containing 2 mL of embryo medium. This experiment was performed in triplicates. To minimize evaporation of the embryo media, the well plates were sealed with parafilm and PVC membrane foil. Plates were maintained at 25 ± 1 °C for 12 days.

The cardiac development in embryos was observed daily from 72 hpf. Heart rhythms were calculated after counting the number of heart contractions over 20 sec. The cardiac development was observed in 5 embryos for each experimental condition.

RNA isolation and cDNA synthesis. On the 12th day larvae were pooled for the total RNA extraction, following the TRIzol™ Reagent protocol. Purity and integrity of the mRNA samples were analyzed using the OD260/280 ratio on a NanoDrop™ 2000/2000c. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcriptation Kit (Applied Biosystems) protocol. Degenerated primers were designed for *cftr* gene by the alignment of the genetic sequences of other fish species, using NCBI's nucleotide sequence library and BioEdit program. Sequences used for the alignment were from *Danio rerio* (NM_001044883.1), *Oryzias dancena* (JQ728537.1), *Oryzias latipes* (XM_004086222.4), *Fundulus heteroclitus* (NM_001309975.1), *Poecilia reticulata* (XM_008410653.2). The primer sequences were: Forward: 5´ TCACCKGTGGARGATGCVAAC 3´; and Reverse: 5´GGCMGACATSAGACTGACSAG 3´.

A PCR was performed using annealing 50°C per 20s and elongation 72°C per 40s, in a cycle of 35 times, the obtained fragment was sectioned from the 1% agarose gel, purified with The Wizard® SV Gel and PCR Clean-Up System protocol and sequenced. It was made a BLAST against GenBank sequences and identified the fragment gene as *cftr*.

rtPCR. The *cftr* primer for real time sequences were: Forward: 5'TTT TGC CTT CTT TGG TGT CC 3'; and Reverse: 5' AGC ATG AAA TGG GTC AAA GG 3'. Primer efficiency 101.06% with R^2 of 0.9979. The *b-actin* primer for real time sequences were: Forward: 5'TGG ACA GGT CAT CAC CAT TG 3'; and Reverse: 5' ACA GGT CCT TAC GGA TGT CG 3'. Primer efficiency 90.86% with R^2 of 0.9997. The process followed the qPCR BIO SyGreen Mix Hi-ROX protocol at QuantStudio3.

Cloning of the *cftr* gene fragment. The RNA was extracted from post-hatch larvae to verify the expression of the *cftr* ion channel, as a measurement of osmotic stress. A fragment of *cftr* and a fragment of the constitutive gene, β-*actin*, were cloned to design specific primers to perform the qPCR analysis. The sequenced fragment from *cftr* had 286bp (GenBank accession number OR853834; Fig. **S1**) and the one from β-*actin* had 372bp (OR853833). It was possible to confirm the identification as the *cftr* gene due to the sequence comparison by BLAST against the GenBank database.

Scanning electron microscope (SEM) analysis. Five embryos were selected and had their chorions cut open slightly by piercing the embryo with a needle under a dissection microscope. Samples were fixed in 2.5% glutaraldehyde solution for 1 h and washed in 0.1M cacodylate buffer, pH 7.2. Then they were post-fixed in 1% osmium tetroxide for 1 h and washed again in cacodylate buffer. Fixed embryos were dehydrated in a series of ethyl alcohol at concentrations from 30% to 70% and stored overnight. The dehydration continued the next day from 80% to 100%. Samples were dried on a Bal-Tec CPD 030 Critical Point Dryer, mounted on a stub and gold covered on the Sputter Coater DSC050. The morphology of the chorion surface and membrane was observed by scanning electron microscope (SEM) (EVO MA10, Zeiss) at 15 kV.

Statistical analysis. Differences in hatching and mortality between treatments were evaluated using one-way ANOVA, with Dunnet´s test post hoc (McGrath, 2011). Two-way ANOVA was used to evaluate heartbeats, followed by Tukey's test. The Shapiro Wilker test was used for checking the normality of the dataset (p- value < 0.05) and the Bartlett test was used for the homogeneity (p-value < 0.05), allowing the use of the ANOVA. All tests were conducted using the R Studio program.

RESULTS

Egg lay in the laboratory and chorion morphology. To obtain eggs at a constant frequency, euryhaline adult fish were kept in brackish water. The egg lay was followed along a period of 52 days and the number of fertilized eggs varied between 100 and 350 (Fig. 1). Near the 15th of each month, there was a peak in the number of eggs.

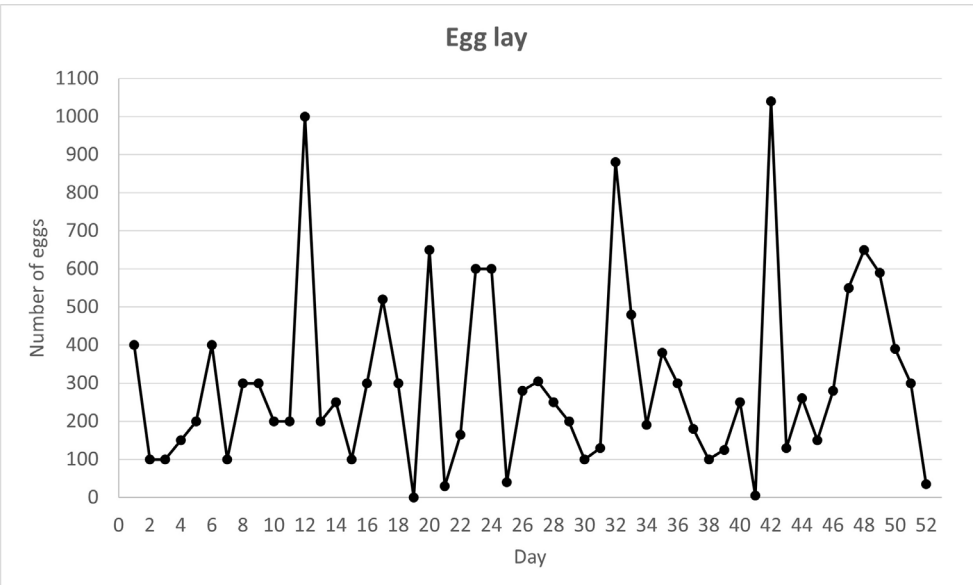

FIGURE 1 | Number of eggs of *Atherinella brasiliensis* laid daily in a period of 52 days from adults maintained in salinity 20 ±1.

The eggs at 3–4 hpf presented a thick chorion with filaments. SEM photos revealed that the chorion structure of Brazilian silverside had several layers, 6–9 µm thick and externally presented a smooth surface with small granules (Figs. 2A, B). The filaments were cylindrical and dense, not hollow (Fig. 2C). The base of the filaments had a ring formation, exactly where it came out of the chorion (Fig. 2D).

Heart rate of embryos raised in different salinities. The heartbeat count started at 96 hpf, since by this time the heart had already developed and could be easily analyzed. Between the 4th and the 6th day, there was no significant variation in the embryo's heartbeat from individuals treated at different salinities (Fig. 3). However, on the 7th day, the heartbeat in all treatments started to increase, and were statistically different compared to the previous day. On the 8th day the rate was almost 20 beats per min higher than on the 7th day. On the 9th day, this increase was even greater, exceeding 170 bpm (Fig. 3). Afterwards, it was not possible to count the heartbeat, as most embryos have hatched and moved continuously under the microscope light. However, it was possible to notice an increase in the number of heartbeats over time until eggs hatched and no differences were observed between individuals treated at different salinities.

Expression of *cftr*. A fragment of *cftr* gene was cloned and sequenced prior to the real-time PCR analysis (GenBank accession number OR853834) (Fig. **S1**). The expression of the *cftr* gene from embryos exposed to different salinities had no significant differences and it was 1.0 ΔΔCt in average when all concentrations were considered (Fig. 4).

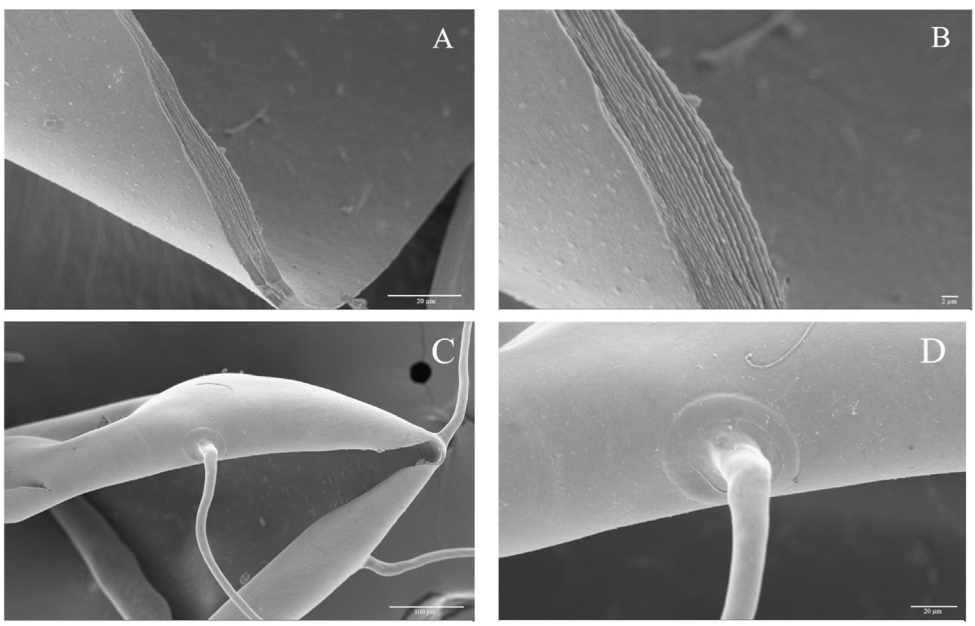

FIGURE 2 | Egg chorion of *Atherinella brasiliensis* seen in scanning electron microscope (SEM). **A.** The chorion of Brazilian silverside composed of several layers. **B.** Detail of the filament layers. **C.** Image of the filament of the chorion. **D.** Detail of the ring formation at the base of the filament.

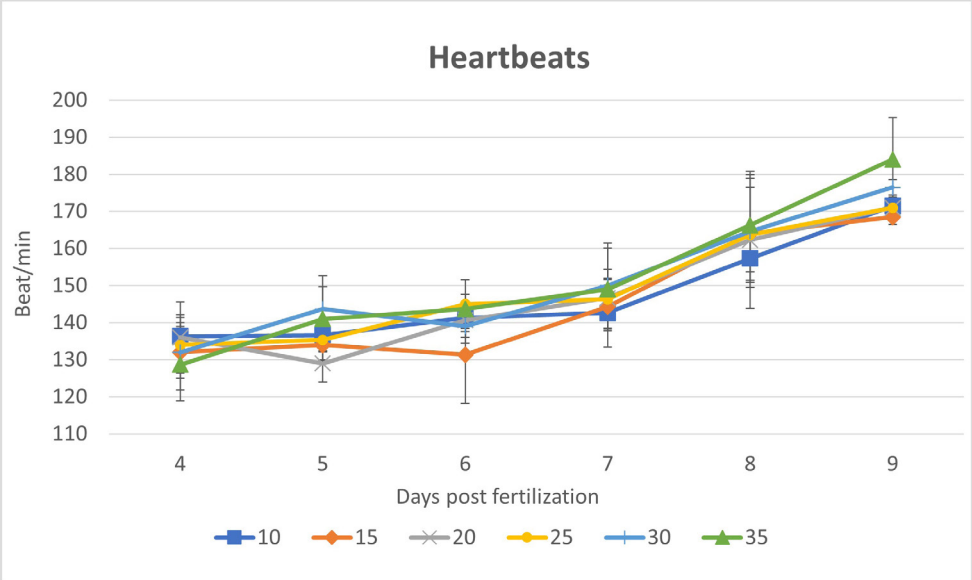

FIGURE 3 | Heartbeats of *Atherinella brasiliensis* embryos between 96hpf and 216hpf raised in salinities from 10 to 35 ($p < 0.05$).

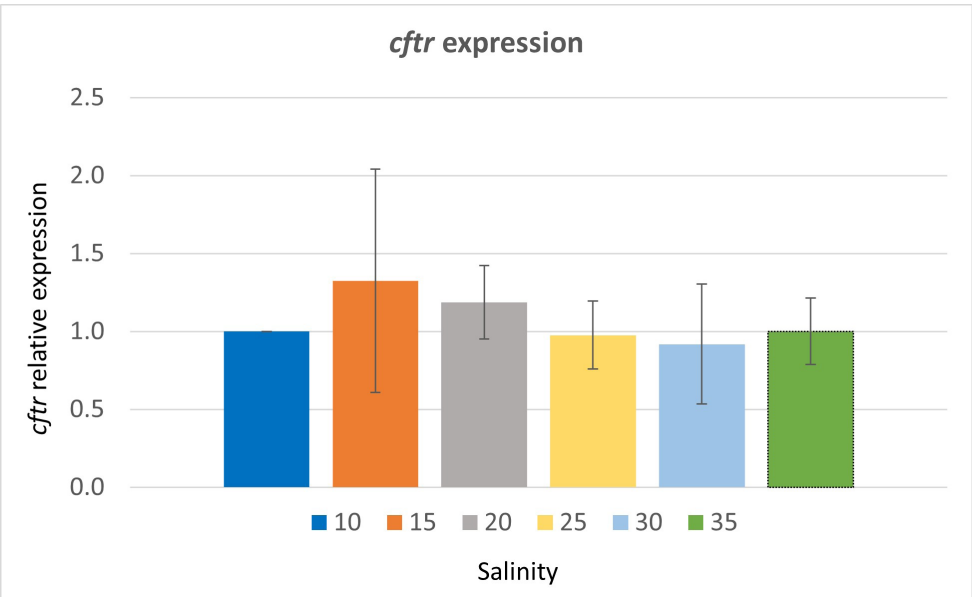

FIGURE 4 | Relative expression of *cftr* in *Atherinella brasiliensis* larvae exposed to salinities 10–35.

DISCUSSION

The eggs at 3–4 hpf presented a thick chorion with filaments similar to medaka embryos (Hart, 1984; Iwamatsu, 2004). The chorion thickness was thinner than the one in medaka eggs (12–15 μm) (Hart *et al*., 1984), but was thicker than the fragile chorion of zebrafish (1.5 μm) (Messaddeq *et al*., 2018). As previously reported, fish eggs that depend on chorion resistance for survival to certain mechanical stress have multilayered and thick chorions (Hart *et al*., 1984; Messaddeq *et al*., 2018) which might represent a physical barrier to large debris.

Regarding the physiological parameters, it was possible to notice an increase in the number of heartbeats over time until eggs hatched and no differences were observed between individuals treated at different salinities, demonstrating that the salinity does not significantly affect the heartbeat frequency during development on the Brazilian silverside. An increase in heart rate until the hatching phase is noticeable in other fish, such as zebrafish and medaka (Gierten *et al*., 2020), and in other vertebrates, such as chicken, lizard, turtle and snake (Tazawa *et al*., 1991; Du *et al*., 2009; Aubret *et al*., 2016).

It is known that the salinity may affect embryonic and larval development of freshwater fish (Hossain *et al*., 2021), and those species cannot be used in toxicity tests of substances at different salinities. Changes in gene expression might indicate a stress condition, and this was not seen in the Brazilian silverside embryos. The *cftr* mRNA levels can change in adults or juvenile of euryhaline fish, when they are acclimated to different salinity conditions (Singer *et al*., 1998; McCormick *et al*., 2003; Scott *et al*., 2004; Lema *et al*., 2018), but not in anadromous stickleback (Taugbøl *et al*., 2014). Disturbances in *cftr* expression might lead to osmoregulatory disfunction, potentially causing oxidative stress. Indeed, oxidative stress can lead to differential expression of CFTR in humans (Zhang *et al*., 2015), or the *cftr* silencing leads to inflammation in zebrafish tissues (Bernut *et al*., 2020). However, during the fish ontogeny not much has been said related to the *cftr* expression, but localization. During the embryo development, the CFTR protein changes its position in ionocytes, and not necessarily its expression. This could be enough to support the osmoregulation in fish, which are therefore able to avoid an osmotic stress (Marshall, Singer, 2002; Bodinier *et al*., 2009). Indeed, early stages of fish can be more resistant to ionic changes, as previously observed for tilapia (Inokuchi *et al*., 2021). Those studies analyzed *cftr* expression and localization after shifts from freshwater to saltwater. Further studies are required to elucidate the c*ftr* expression in different tissues and during the transition between freshwater to saline water. In addition, the investigation with other osmoregulatory transporters, such as NKA-ATPase and H+-ATPase, are necessary to assess the metabolic involvement of transporters.

Brazilian silverside embryos presented constant physiological parameters at different salinity conditions, such as heart rate and the expression of *cftr* gene, suggesting that embryos might not be under osmotic stress at the gene expression level. Altogether the obtained results revealed that the Brazilian silverside embryos did not show signs of stress during ontogeny when exposed to a wide range of salinities. Therefore, this species is suitable for studying ionocytes mechanisms, membrane transporter ion channels during different life stages, and also for embryology testing in environmentally relevant conditions.

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AUTHORS' CONTRIBUTION

Carolina Brioschi Delpupo: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft.

Chris I. Espeland: Data curation, Formal analysis, Investigation.

Aline Karl Araújo: Data curation, Investigation, Methodology.

Jackson de Souza-Menezes: Formal analysis, Investigation, Supervision, Writing-original draft.

Daniela M. Pampanin: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing-original draft, Writing-review and editing.

Natália Martins Feitosa: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Validation, Writing-original draft, Writing-review and editing.

ETHICAL STATEMENT

Animals were handled and experimented according to the protocols of the Institutional Animal Care and Use Committee of the Universidade Federal do Rio de Janeiro under number 063/17.

COMPETING INTERESTS

The author declares no competing interests.

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