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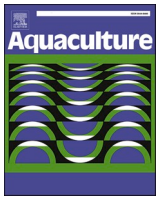

Survival of Atlantic bluefin tuna (*Thunnus thynnus*) larvae hatched at different salinity and pH conditions

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ABSTRACT

In this study, we assessed the effect of environmental salinity and pH as independent factors on larval survival of Atlantic bluefin tuna (ABFT –*Thunnus thynnus*) together with their whole-body Na^+/K^+-ATPase and v-type H^+-ATPase activities. Fertilized eggs of ABFT were obtained from a spontaneous spawning of broodstock in the farming facilities at El Gorguel (Cartagena, SE Spain) and were transferred to facilities of the Spanish Institute of Oceanography (IEO) in Mazarrón (SE Spain). In a first experiment, eggs (200 fertilized eggs L^{-1} per treatment, in 3 replicates) were exposed to different salinities treatments and constant pH 8.0 (control) until hatch was completed (50 h post-fertilization, hpf, at 23 °C): 27, 30, 33, 36, 37, 38 (control), 39, 40, 43, 46 and 49 ppt. In a second experiment eggs (200 fertilized eggs L^{-1}, in 3 replicates) were exposed to seawater salinity (SW: 38 ppt) and four reduced pH treatments until hatch was completed (50 hpf at 23 °C): 8.0 (control), 7.7, 7.5 and 7.3. An inverse "U-shaped" relationship was observed between environmental salinity and number of hatched larvae. An opposite pattern was observed for both Na^+/K^+-ATPase and H^+-ATPase activities in hatched larvae, increasing both activities in groups exposed to extreme salinities. Thus, larval survival was higher at intermediate salinities and lower at the extreme salinities tested. These results suggest higher survival rates with lower active pumps activities. No significant differences in larval survival were observed with pH treatment, but lower H^+-ATPase activity was detected at control environmental pH (pH 8.0). Survival results are discussed in terms of osmoregulatory cost adapting to a salinity and pH predicted for the near future scenarios.

1. Introduction

Capture of Atlantic bluefin tuna (ABFT, *Thunnus thynnus*) is strictly regulated internationally due to a sharp decline in its wild populations (ICCAT, 2021). Developing aquaculture for this species seems reasonable in the near future to meet market demand. However, although advances have been made in its domestication, their reproduction in captivity is still a bottleneck (Mylonas et al., 2007). In this sense, it is interesting to know that ABFT are capable of spawning during at least two subsequent reproductive seasons while maintained in sea cages (Medina et al., 2016) offering a source of fertilized eggs for cultivation. On the other hand, one of the major challenges of tuna aquaculture is to increase survival during the first 10 days after hatching, as described in *T. orientalis* (Sawada et al., 2005).

In wild spawning grounds, environmental conditions are involved in the distribution of ABFT eggs and larvae (Ingram et al., 2017; Reglero et al., 2017). Some of these physico-chemical parameters are of special relevance during the first stages of life of tunas, affecting their survival (Kimura et al., 2010). In this way, temperature, salinity and water pH are amongst the most relevant physico-chemical variables in larval survival of *Thunnus spp.* (Kimura et al., 2010; Tsuda et al., 2012). While temperature has a direct relationship with the activity of enzymatic

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processes, being closely related to larval growth of *T. atlanticus* (Gleiber et al., 2020), the effect of water salinity and pH present physiological bases related to metabolic and osmotic homeostasis. Environmental conditions seem to be more limiting in ABFT during its early stages of life, since adults and sub-adults make great migrations through the Mediterranean and the Atlantic (Aranda et al., 2013; Varela et al., 2020), with highly variable salinity, temperature and pH conditions. Thus, long-term changes in water salinity (of about 0.3–0.6 ppt / 100 yr) have been described in the spawning area of ABFT in the Mediterranean (Vargas-Yáñez et al., 2021), while ocean acidification was also reported in spawning grounds of other tuna species, such as *T. albacares* (Nicol et al., 2022).

Marine teleosts have lower concentration of ions in their internal fluids compared to the surrounding seawater (Evans et al., 2005). Therefore, there is an osmoregulatory effort by certain tissues, such as the gills, intestine or skin to excrete NaCl against a gradient in order to maintain internal fluids stable (Laiz-Carrion et al., 2005; Ruiz-Jarabo et al., 2017; Ruiz-Jarabo et al., 2021). These processes, in adult *T. albacares* and skipjack tuna (*Katsuwonus pelamis*) are estimated to cost between 9 and 13% of the standard metabolic rate (SMR) (Brill et al., 2001), while in other teleosts the costs could be as high as 20% of the SMR (Heuer and Grosell, 2016) or even 50% of the total fish energy budget (Boeuf and Payan, 2001). In addition, studies conducted in several teleost species, including *T. albacares*, revealed the importance of osmoregulatory organs during early stages of development, and highlighted the involvement of ATPase-dependent ion transporters located in ionocytes of this species (Kwan et al., 2018).

Environmental salinity is thus of relevance for the proper development and survival of fish larvae due to the associated costs in osmoregulation (Ruiz-Jarabo et al., 2015). To date, information regarding the effect of salinity on ABFT is scarce, and limited to descriptions of the environmental conditions of the spawning grounds (Alemany et al., 2010), while it has been stated that salinities below 34 ppt increased juvenile *T. orientalis* mortality (Tsuda et al., 2012). The interest for the aquaculture sector in establishing safe environmental salinity limits to cultivate ABFT is evident, since it has a direct impact on the survival rates of the species. However, it is not always possible to keep the environmental conditions stable throughout the year, or the salinity may not be the same as where spawning occurs in wild animals. Thus, to establish an adequate ABFT culture it is necessary to first describe the optimal salinity for hatching and larval survival, which to date is unknown.

Water acidification is also a matter of concern for global aquaculture. Ocean acidification produces imbalances in the acid-base homeostasis of teleost fish, affecting energy management and growth (Heuer and Grosell, 2016; Ruiz-Jarabo et al., 2021) due to increased osmoregulatory costs (Alves et al., 2020; Gregorio et al., 2019). Evidences suggested that recruitment of marine tuna species, specially eggs and larvae, are affected by ocean acidification through changes in water pH, as seen in *T. albacares* (Bromhead et al., 2015; Nicol et al., 2022). However, there is no information on the effect of water acidification on the hatching or survival rates of ABFT larvae.

The aim of this study is to describe the effect of water salinity and pH on hatching rates and larval survival of ABFT. The experimental range to be tested goes from 27 to 49 ppt salinity, in accordance to the best growing rates of other teleosts (Boeuf and Payan, 2001; Ruiz-Jarabo et al., 2015) and the oceanic life of this species (Alemany et al., 2010); and from the current oceanic pH values of 8.0 to pH 7.3, which is the expected drop due to ocean acidification by the year 2300 (Caldeira and Wickett, 2003). Assuming that inappropriate environmental conditions of these variables will produce osmoregulatory imbalances, and a subsequent energy costs enhancement, the activity of relevant enzymes (Na^+/K^+-ATPase and v-type H^+-ATPase) in this process are also analysed. This will serve to highlight whether a low survival rate is related to osmoregulatory overstrain.

2. Material and methods

2.1. Eggs obtainment

Fertilized eggs of ABFT were obtained on 25th June 2016 (at 3.00–4.00 UTC) from a spontaneous spawning of broodstock in the farming facilities at El Gorguel (37° 33′ N, 0° 52′ W, Cartagena, SE Spain) of Caladeros del Mediterráneo Company. Salinity, pH and temperature conditions of seawater were 38 ppt, pH 8.0 and 23.0 °C at the moment of fertilization. Fertilized eggs were transferred to facilities of the Spanish Institute of Oceanography (IEO) in Mazarrón (SE Spain) after 5 h post-fertilization (hpf), according with the procedure previously described (de la Gándara et al., 2016). All animal experiments were performed in agreement with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals. Animal experimentation and analysis was restricted to the first 50 hpf.

2.2. Experimental conditions

Fertilized eggs (5 hpf) were introduced into 250-mL aquaria ($n = 50$ per aquaria) and kept without water renewal and under natural photoperiod conditions until complete hatching. Oxygen levels were monitored and during the whole experiment were maintained above 6.00 mg L^{-1} (Oxymeter, Oxyguard). In a first experiment, fertilized eggs ($n = 150$ per treatment, in 3 replicates) were exposed to a range of environmental salinities until hatch was completed (50 hpf at 23 °C): 27, 30, 33, 36, 37, 38 (control), 39, 40, 43, 46 and 49 ppt. Water pH in all salinities was 8.0. Experimental salinities were achieved by mixing full-strength seawater with distilled water or by mixing seawater (SW: 38 ppt) with natural marine salt (Salinera Española, San Pedro del Pinatar, Murcia, Spain). In a simultaneous second experiment, fertilized eggs (n = 150 per treatment, in 3 replicates) were exposed to SW salinity (38 ppt) and four pH treatments for 44 h at 23 °C: 8.0 (control), 7.7 (near future), 7.5 (far future) and 7.3 (lowest pH treatment) according to current ocean predictions (Caldeira and Wickett, 2003; Hoegh-Guldberg et al., 2014). The pH manipulation was performed by dissolving very small amounts of pure CO_2 (99.9% purity) with fine diffusers in the treatments directly into the experimental aquaria water. This addition was performed manually at the beginning of the experiment and was monitoring every sampling point, in order to maintain the levels of pH in the acidified tanks accordingly to each treatment (8.0, 7.7, 7.5, and 7.3). No pH changes were observed and no water changes were needed during the experiment. The lowering of pH was closely monitored using a pH meter/glass electrode (HI-1131B, Hanna Instruments, Rhode Island, United States), which was calibrated on a daily basis with certified reference buffers in the NBS scale (NIST-traceable pH buffer solutions 4.01, 7.00 and 9.21, Crison Instruments, Barcelona, Spain), following standard procedures (Dickson et al., 2007). The NBS scale was chosen knowing that there may be slight discrepancies in pH due to interaction with other ions in seawater, but this selection allows us to make comparisons with previous studies carried out by our and other research groups (Alves et al., 2020; Ruiz-Jarabo et al., 2021). In order to mimic the physical perturbation associated with CO_2 bubbling, the control tanks were also bubbled with similar small amounts of compressed air at current atmospheric CO_2 concentrations. During the experiment, and in order to preserve water quality, non buoyant opaque eggs were removed from the aquariums and counted as dead. No changes were observed in water pH or salinity during the experiment.

2.3. Sampling

At time 40 hpf eggs started to hatch, and complete hatching occurred about 45 hpf. Just hatched larvae float close to surface responding to some physical stimuli. All alive and dead larvae, as well as dead eggs were counted at the end of the experimental time (50 hpf). Percentage of

hatched larvae (both alive and dead at the end of the experiment) and total survival rate (percentage of alive larvae at the end of the experiment with respect to the total number of fertilized eggs at the beginning) were calculated. At this time, alive larvae from each aquarium were introduced into three 3 mL vials containing 1 mL ice-cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) for the analysis of ATPase enzymes activity, and snap frozen in liquid nitrogen. Hypothermic shock appears to be an effective method for euthanasia in fish larvae (Strykowski and Schech, 2015). Samples were maintained at −80 °C until their analysis.

2.4. Enzyme activities

Na^+/K^+-ATPase (NKA) activity in single whole-larvae homogenates were determined in microplates using a modification (Mancera et al., 2002) of McCormick's method (McCormick, 1993) with 0.5 mM ouabain (O3125; Sigma, Spain) as a specific inhibitor of 100% NKA activity. Vacuolar-type H^+-ATPase (HA) activity was analysed as described before (Ruiz-Jarabo et al., 2016), using 100 nM bafilomycin A1 (B1793; Sigma, Spain) as a specific inhibitor of the HA. The reactions were allowed to proceed at 25 °C. All enzyme activities were determined using a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft® Windows XP. Reaction rates of enzymes were determined by changes in absorbance from the oxidation of NADH to NAD^+, measured at 340 nm. Data were expressed as μmol ADP $larvae^{-1}$ h^{-1}.

2.5. Statistics

Normality and homogeneity of variances were analysed using the Shapiro-Wilk's test and the Levene's test, respectively. Differences between groups were tested using one-way ANOVA with salinity (27, 30, 33, 36, 37, 38, 39, 40, 43, 46 and 49 ppt) or pH (8.0, 7.7, 7.5 and 7.3) as independent variables. When necessary, data was logarithmically transformed to fulfil the requirements for ANOVA. Tukey *post hoc* test was used to identify significant differences between groups. Statistical significance was accepted at $p < 0.05$. All the results are represented as mean ± SEM. All tests were performed using Statistica 7 software for Windows.

3. Results

3.1. Salinity

The effects of environmental salinity on the number of hatched larvae and their total survival is shown in Fig. 1. The highest hatching rates occurred within the range between 33 and 49 ppt salinity (at pH 8.0, normal SW pH), averaging 84.0 ± 3.5%, decreasing dramatically at the lowest salinity tested (27 ppt), with 55.9 ± 9.3% ($p < 0.05$, one-way ANOVA followed by a *post hoc* Tukey test). Larval survival showed an inverse "U shape" curve respect to environmental salinity, so the range showing the highest total survival rates covered the environmental salinities from 33 to 40 ppt, averaging 79.7 ± 3.8% survival, while total survival significantly decreased at the extremes, reaching 37.8 ± 13.5% at 27 ppt and 61.6 ± 1.8% at 49 ppt salinity ($p < 0.05$).

Influence of salinity on the Na^+/K^+-ATPase (NKA) and the v-type H^+-ATPase (HA) activities are shown in Fig. 1, observing a clear "U-shaped" relationship between environmental salinity and both enzymatic activities. The lowest NKA activities were described at 36 and 37 ppt salinity (88 ± 18 and 60 ± 11 μmol ADP $larvae^{-1}$ h^{-1}, respectively),

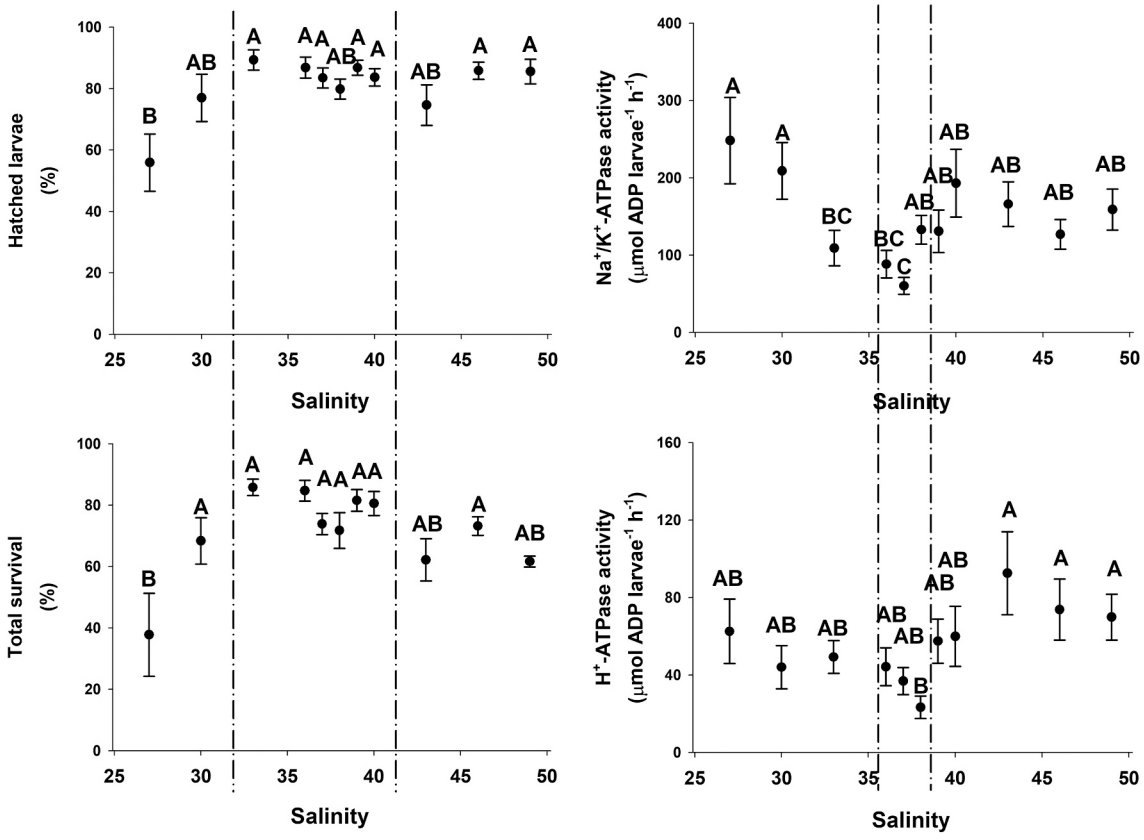

Fig. 1. Hatching and total survival rates (left) and Na^+/K^+-ATPase and v-type H^+-ATPase activities (right) of Atlantic bluefin tuna eggs maintained at different environmental salinities. Sampling was done at 50 hpf (hours post fertilization). Values are mean ± SEM (standard error of the mean). Different letters indicate significantly different groups ($p < 0.05$, one-way ANOVA followed by a *post hoc* Tukey test). Vertical lines delimit the range of salinities with the highest hatching/survival rates and lowest ATPase enzymes activities.

while the highest were observed at the extreme salinities tested. Thus, at 27–30 ppt, NKA averaged activity was 229 ± 46 μmol ADP larvae $^{-1}$ h^{-1}, and it was averaged at 161 ± 30 μmol ADP larvae $^{-1}$ h^{-1} within the range from 40 to 49 ppt salinity. Extreme environmental salinities thus showed statistically different NKA activities than larvae maintained at 36–37 ppt ($p < 0.05$). The lowest HA activities were described at 37 and 38 ppt salinity (37 ± 7 and 23 ± 6 μmol ADP larvae $^{-1}$ h^{-1}, respectively), while the highest were observed at the highest salinities (43 to 49 ppt), averaging 79 ± 16 μmol ADP larvae $^{-1}$ h^{-1}, with statistical differences between groups at 37–38 ppt and 43–49 ppt ($p < 0.05$).

3.2. pH

The effects of environmental pH on the number of hatched larvae and their total survival are shown in Fig. 2. The effects of pH treatment at 38 ppt salinity (normal SW salinity) on hatching and total survival were not significant in this study ($p > 0.05$, one-way ANOVA followed by a *post hoc* Tukey test). Thus, averaged hatching rate within the range from pH 7.3 to pH 8.0 was 82.8 ± 4.0%, while total survival rate was 73.8 ± 3.9%.

Influence of pH on NKA and HA activities are shown in Fig. 2. The lowest NKA activity occurred at pH 7.7 (80 ± 14 μmol ADP larvae $^{-1}$ h^{-1}), being statistically different than NKA activity at the extremes, with 130 ± 9 and 133 ± 19 μmol ADP larvae $^{-1}$ h^{-1} at pH 7.3 and pH 8.0, respectively ($p < 0.05$). HA activity showed its significantly lowest values at pH 8.0 (23 ± 6 μmol ADP larvae $^{-1}$ h^{-1}) and a gradual increase towards more acid environments, reaching 62 ± 18 and 57 ± 26 μmol ADP larvae $^{-1}$ h^{-1} at pH 7.3 and 7.6, respectively ($p < 0.05$).

4. Discussion

The present study evidenced that higher hatching rates and total survival of Atlantic bluefin tuna (*Thunnus thynnus*) larvae occurs within the range 33–42 ppt salinity, while there are no differences due to SW pH within the range studied from 7.3 to 8.0 (at 38 ppt environmental salinity). This could be a consequence of extra energy costs due to osmoregulation in extreme salinities, as seen by the high activities of Na^+/K^+-ATPase (NKA) and v-type H^+-ATPase (HA) enzymes in whole ABFT larvae. These results are of interest for the improvement of ABFT aquaculture, as mortality in early life stages due to inappropriate environmental conditions could compromise the production in captivity.

4.1. Environmental salinity

Under the environmental conditions described herein, ABFT shows 80 to 89% hatching rates within a range of salinities from 33 to 42 ppt, with larvae showing 90 to 97% survival after hatching. These rates are higher than those of *T. orientalis* 1 day post hatching (dph), which are around 80% in control SW (Kimura et al., 2010). However, previous experiments with ABFT show a survival rate of 53% at 20 dph (Reglero et al., 2014), highlighting the massive mortalities of this species during its early development. Therefore, the search for suitable physico-chemical conditions, which increase initial survival, may be crucial to improve the crop yield of this species. In this line, some physiological approaches may help to determinate the best salinity conditions for ABFT larvae.

In this way, as osmoregulation is an energetically expensive process in tunas (Brill et al., 2001), the analysis of NKA and HA activities may serve as energy management biomarkers to highlight the most favorable environmental salinity in terms of osmoregulation. Our results indicate

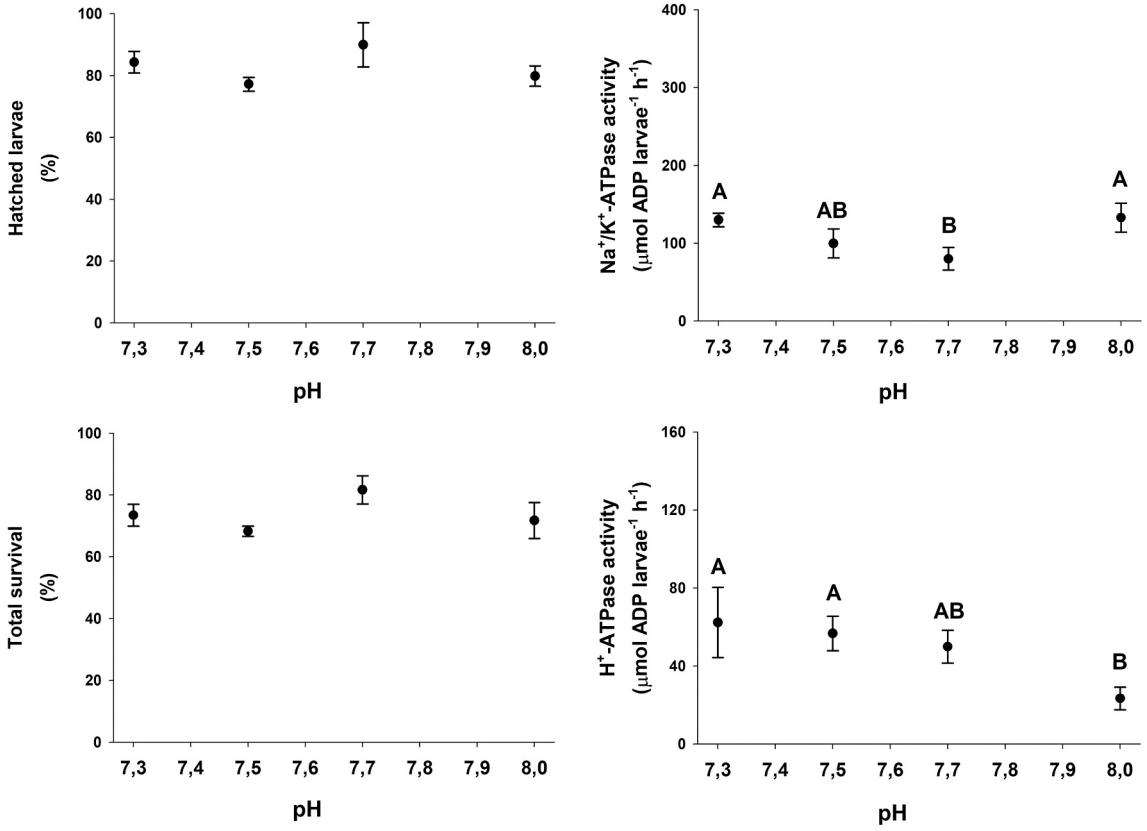

Fig. 2. Hatching and total survival rates (left) and Na^+/K^+-ATPase and v-type H^+-ATPase activities (right) of Atlantic bluefin tuna eggs maintained at different environmental pH. Sampling was done at 50 hpf. Values are mean ± SEM (standard error of the mean). Different letters indicate significantly different groups ($p < 0.05$, one-way ANOVA followed by a *post hoc* Tukey test).

that the lowest whole-larvae NKA and HA activities occur within a tight range between 36 and 38 ppt salinity. Metabolic rates of ABFT larvae are higher than those of other active scombrids (Blanco et al., 2020), which is in line with the higher oxygen demand of adult ABFT compared to other marine fish species (Korsmeyer and Dewar, 2001). In adult tunas, anaerobic metabolism in white muscle is of major importance, showing the highest lactate levels in all studied teleosts (Arthur et al., 1992). As higher lactate oxidation in gill cells is correlated to higher branchial NKA activity (Perry and Walsh, 1989), and due to the great similarities between gill and cutaneous ionocytes in *T. albacares* larvae (Kwan et al., 2018), it is reasonable to assume that both cell types can be fueled by lactate mobilized from the muscle. Thus, according to our results, we suggest that ABFT larvae faced a great energy demand to satisfy cutaneous ionocytes, before full development of gills (Brauner, 2008), requirements at extreme salinities higher than 42 ppt and below 33 ppt (where higher mortality rates are observed). In this sense, lecitotrophic fish larvae, that have not yet begun to eat autonomously, present a certain amount of energy reserves. Due to the exponential growth rates of tunas during their first life stages (Blanco et al., 2017), the amount of required energy to support this body mass gain could be compromised when osmoregulatory costs, imposed by inappropriate environmental conditions, are high. Our hypothesis is that under extreme salinities where NKA and HA activities are higher, ABFT early stages consume a sufficient amount of energy to cope with the osmoregulatory stressor, which inevitably leads to a higher mortality rate due to exhaustion. On the other side, the minimum whole-body NKA and HA activities are observed in ABFT larvae in a narrow range between 36 and 38 ppt salinity. Conspicuously, ABFT spawning in the Balearic Islands (from whose reproducers the eggs of the present study were obtained) preferred waters with salinities between 36.9 and 37.5 ppt (Alemany et al., 2010). This data, together with the results obtained in the present study, may suggest that this is the optimal environmental salinity range (at least for the larvae obtained from the breeding stock of this geographic area) for the cultivation of the first life stages of ABFT due to the lower costs of osmoregulation. However, further studies are necessary, including long-term culture at different salinities of larvae and juveniles to check for differences in growth rates.

4.2. Environmental pH

Seawater acidification in this study do not affect egg hatching, or larvae survival. This may be related to the lack of differences in whole-body NKA activity in ABFT larvae of 50 hpf, highlighting there are no costly energy processes related to osmoregulation. However, a HA activity enhancement was observed at lower water pH conditions. Branchial v-type H^+-ATPase activity increased in *Salmo salar*, *Galaxias maculatus* and *Solea senegalensis* (some aquacultured species) at low environmental salinities as part of the ion capture process in hyposmotic waters (Bystriansky and Schulte, 2011; Ruiz-Jarabo et al., 2017; Ruiz-Jarabo et al., 2016). However, during the acclimation to ocean acidification, this enzyme collaborates, through different processes, in the recovery of the imbalances caused by the observed drop in blood pH (Ruiz-Jarabo et al., 2021). Plasma acidosis, in *Sparus aurata* and *Dicentrarchus labrax* is counteracted through intestinal secretion of bicarbonate, forcing the animal to increase its drinking rates and intestinal osmoregulatory processes, leading to a higher energy expenditure and lower growth rates (Alves et al., 2020; Gregorio et al., 2019; Ruiz-Jarabo et al., 2021). The excess of protons in blood of fish during seawater acidification is excreted through H^+ transporters, including HA, located in apical membranes of osmoregulatory cells (Heuer et al., 2016; Tresguerres and Hamilton, 2017). Even though ocean acidification effects larval stages have not been studied in detail yet in tunas (Llopiz et al., 2014), we hypothesize that in the long term, ABFT larvae kept in an acidic environment will grow less than those at a pH close to 8. Further larval rearing experiments will be necessary to corroborate this hypothesis.

4.3. Future environmental scenario

Concentrations of CO_2 in the ocean tend towards equilibrium with the CO_2 in the atmosphere, causing a gradual increasing CO_2 absorption by the oceans and changing substantially the ocean water chemistry, specifically by increasing concentrations of dissolved carbon dioxide, carbonic acid, bicarbonate ions and hydrogen ions (H^+), and decreasing concentrations of carbonate ions (Fabry et al., 2008). Ocean acidification might be a concern for marine fishes, disturbing the acid–base regulation, blood circulation, and respiration, as well as the nervous system, leading to long-term effects such as reduced growth rates and reproduction (Portner et al., 2004).

Other direct effects of ocean acidification have been found in behavior (Dixson et al., 2010; Ferrari et al., 2011; Munday et al., 2009), development (Frommel et al., 2011; Munday et al., 2009), RNA/DNA ratios (Franke and Clemmesen, 2011), as well as otolith growth and development (Checkley Jr. et al., 2009; Munday et al., 2011b; Munday et al., 2011a) of marine fish larvae. Elevated CO_2 concentrations can alter acid-base balance directly in the control of internal pH through the active and energetically expensive proton transport pumps (v-type H^+-ATPases), and indirectly by other salt balance trough ion pumps (Na^+/K^+-ATPases) and transport channels (Heuer and Grosell, 2014; Kreiss et al., 2015; Marshall and Grosell, 2005). The energy cost enhancement due to higher active pumps activities, to cope with of acidification stress, often combined with higher salinity and temperature, could be crucial in these highly-energy demand early life stages with an exponential growing rate, producing lower survival potential (Brill et al., 2001). In addition, these adverse conditions will induce slower-growing individuals that will spend more time in this stages that are particularly vulnerable to mortality *via* starvation and predation (Cowan and Shaw, 2002; Houde, 1987). Because of their limited capacity for ion exchange, embryos and larvae of marine fishes are predicted to be more sensitive to elevated CO_2 than juveniles and adults (Brauner, 2008). Also, depending on their habitat needs, species with specific habitat requirements for spawning or nursery grounds display bottlenecks in their life cycle more affected by ocean acidification (Shen et al., 2017).

In conclusion, ABFT eggs show higher hatching rates and larval survival at 36–38 ppt salinity, coinciding with the lowest whole-body NKA and HA activities. Furthermore, although survival rates do not seem to change due to ocean acidification (within the range from pH 8.0 to 7.3), the increase in HA activity at lower pH may indicate a long-term decline in the growth rate of larvae. This study serves as a basis to further explore the effect of environmental aquaculture conditions of ABFT during its early life stages.

Author statement

The authors declare that no informed consent was required for experimentation with human subjects in this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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