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First estimates of metabolic rate in Atlantic bluefin tuna larvae

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Abstract

Atlantic bluefin tuna is an iconic scombrid species with a high commercial and ecological value. Despite their importance, many physiological aspects, especially during the larval stages, are still unknown. Metabolic rates are one of the understudied aspects in scombrid larvae, likely due to challenges associated to larval handling before and during respirometry trials. Gaining reliable estimates of metabolic rates is essential to understand how larvae balance their high growth needs and activity and other physiological functions, which can be very useful for fisheries ecology and aquaculture. This is the first study to (a) estimate the relationship between routine metabolic rate (RMR) and larval dry weight (DW) (mass scaling exponent) at a constant temperature of 26°C, (b) measure the RMR under light and darkness and (c) test whether the interindividual differences in the RMR are related to larval nutritional status (RNA/DNA and DNA/DW). The RMR scaled nearly isometrically with body size (b = 0.99, 0.60–31.56 mg DW) in contrast to the allometric relationship observed in most fish larvae (average b = 0.87). The results show no significant differences in larval RMR under light and darkness, suggesting similar larval activity levels in both conditions. The size explained most of the variability in RMR (97%), and nutritional condition was unrelated to the interindividual differences in routine metabolism. This is the first study to report the metabolic rates of Atlantic bluefin tuna larvae and discuss the challenges of performing bioenergetic studies with early life stages of scombrids.

KEYWORDS
Atlantic bluefin tuna, larvae, metabolism, nutritional condition, respiration, RMR

1 | INTRODUCTION

Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus, 1758) is one of the most valuable pelagic resources worldwide (Juan-Jordá *et al*., 2013). Despite the recent success closing its life cycle in captivity (Ortega & de la Gándara, 2017), there are still several factors limiting the aquaculture production of this species. Mortality during the early life stages is still a clear bottleneck to upscale production. Knowledge of

the physiology of these early life stages can help identify vulnerabilities during ontogeny and increase larval survival. For example, increases in oxygen consumption have been reported during times of high metabolic demand, such as changes in the morphology, physiology and behaviour of Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844) larvae (Miyashita *et al*., 1999). Knowing when those changes take place during the larval stage and measuring the energy expenditure can help improve larviculture protocols by optimizing necessary daily nutritional requirements. In addition, translating larval physiological knowledge into predictive models might improve the understanding of species responses to perturbations and develop more reliable indices of annual recruitment (Jørgensen *et al*., 2016; Peck *et al*., 2013; Reglero *et al*., 2019, 2018).

Metabolism is the sum of the processes by which energy and materials are transformed into various living structures and activities (Gillooly *et al*., 2001). Metabolism supports not only cellular and tissue maintenance but also additional vital activities, indicating how organisms partition energy resources to activities that allow them to survive, grow and reproduce (Post & Lee, 1996). Metabolic rate (M) changes with body size (B) following the equation $M = aB^b$. The mass scaling value (slope, b) ranges from 0.6 (allometric relationship) to 1 (isometric relationship) (*e.g*., allometry, Finn *et al*., 2002; isometry, Giguere *et al*., 1988) and varies across species, life stages, lifestyles and habitats (Killen *et al*., 2010; Kiørboe & Hirst, 2014). One example is the species shorthorn sculpin *Myxocephalus scorpius* (Linnaeus, 1758) that has a slope, b, higher than 1 during the pre-metamorphic stages and decreasing to 0.8 in later life stages (Killen *et al*., 2007). Quantifying the changes in metabolic rates during ontogeny can help identify key changes in energy demands and compartmentalization.

The measurements of metabolic rates in the early life stages of marine fish are still relatively scarce compared to the later stages, probably due to their small size, high sensitivity to stress from handling and confinement and the absence of standard equipment for these stages (for a review, see Peck & Moyano, 2016). Measurements in fish larvae are commonly performed *via* oxygen consumption in closed respirometry (Nelson, 2016). The routine metabolic rate (RMR) is likely the most common metabolism measurement done in the early stages of fish. It represents the minimum maintenance costs [standard metabolic rate (SMR)] and also includes some degree of activity (Houde & Schekter, 1983; Peck & Buckley, 2008).

Understanding the energy balance between growth and metabolism in the early life stages of warm water species, such as scombrids, is especially relevant as they need to support growth rates exceeding 30% per day (Houde, 1989; McCarthy & Fuiman, 2011). To the authors' knowledge, metabolic rates have never been estimated in larvae, juveniles or adults of Atlantic bluefin tuna. In Pacific bluefin tuna, only one study has examined ontogenetic changes in oxygen consumption during the larval stage (Miyashita *et al*., 1999). These authors observed that oxygen consumption and metabolic activity rapidly increased in the period between hatching and juvenile metamorphosis and that these metabolic costs were significantly higher than those of other species at the same temperatures. These high costs were related to their fast developmental and activity rates. Before the third week of life, Atlantic bluefin tuna larvae develop an adult-type digestive system and most juvenile morphological characteristics (Reglero *et al*., 2014; Yúfera *et al*., 2014). The sensitive nature and the high activity of tuna larvae are problematic when performing respirometry tests in confinement. One solution to reduce this confinement stress, not yet tested in larval stages, is running the respirometry measurements in darkness. Because these larvae, as most marine larvae, are visual feeders (Margulies, 1997), one expects that they largely reduce their activity in darkness. In fact, this method has been previously used in the larvae of other species as a proxy for SMR (Finn *et al*., 1995, 2002; Porter, 2001).

The nutritional condition of marine fish reflects the energy reserves available for maintenance, growth and activity, and the individual responses to variability in food supply and feeding success are ultimately related to survival (Anderson, 1988; Cushing, 1990; Hjort, 1914). This study used two different indices to determine the larval nutritional condition: the RNA/DNA and the DNA/DW (dry weight). The RNA/DNA is an index of cell metabolic intensity, and it is used as an approach for recent growth and recent nutritional condition of fish larvae (Clemmesen, 1994; Folkvord *et al*., 1996). The amount of DNA is stable under changing environmental situations reflecting the number of cells of an individual, whereas the amount of RNA is directly proportional to the protein synthesized in the cell which is highly dependent on food quantity/quality (Buckley *et al*., 1999). Therefore, well-fed larvae, metabolically more active, grow faster and have relatively higher RNA/DNA compared to poor-fed larvae with less-active metabolism (Clemmesen, 1987, 1994). On the contrary, the DNA/DW show larval cell condition, increasing when the condition decreases, because less weight is attributed per cell number (Bergeron, 1997).

The present study reports the first estimates of RMR in Atlantic bluefin tuna larvae. The main objectives of this study are to (a) estimate the relationship between RMR and the larval DW (mass scaling exponent) at 26°C, (b) measure RMR in light and darkness and (c) test whether the interindividual differences in RMR are related to larval nutritional status. Understanding how metabolic rates vary in Atlantic bluefin tuna is very useful to estimate how they balance and adapt growth and activity during the fast-growing larval period.

2 | MATERIALS AND METHODS

2.1 | Ethical standards

The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and Spanish directive RD53/2013. All specimens studied were handled in accordance with the Guidelines of the Bioethical Committee of the Instituto Español de Oceanografía (IEO) (reference REGA ES300261040017), and the protocol was approved by the Committee on the Ethics of Animal Experiments of the IEO (permit number: 2017/01).

2.2 | Larval rearing

Fertilized Atlantic bluefin tuna (ABFT) eggs were obtained from naturally spawning captive adults in the farming facilities at El Gorguel, Cartagena (southeast Spain), owned by Caladeros del Mediterráneo S.L. The eggs were collected and transported to the Spanish Institute of Oceanography (IEO) facilities in Mazarrón (southeast Spain) where the experiment was performed. Approximately 50,000 eggs were incubated in a 5000 l tank (assuming 85%–90% hatching success, 8.5–9 larvae per litre), where the larvae hatched and remained until 26 days post-hatching (dph). At 26 dph all the larvae were removed from the tank (n = 203), the smallest 100 larvae (10.10 ± 2.80 mg) were selected and transferred to a new 1500 l rearing tank and the rest (22.80 ± 4.70 mg) were removed for weaning proposes and were not used in this experiment. Every day, groups of 7–15 larvae were randomly sampled for metabolism from the 5000 l tank (17–26 dph) or from the 1500 l tank (27–30 dph). During the larval rearing period, the photoperiod regime was 14 h of light and 10 h of darkness, 14lL:10D. The larvae were reared at an ambient temperature (24°C–26°C) and a salinity of 38. Prey were always *ad libitum* in the tanks, and the feeding schedule consisted of (a) enriched rotifers *Brachionus plicatilis* (Müller, 1786) (4–16 dph), (b) enriched *Artemia* nauplii *Artemia franciscana* instar II (Kellogg, 1906) (AF, INVE AQUACULTURE, Dendermonde, Belgium) (11–23 dph) and (c) gilthead seabream *Sparus aurata* (Linnaeus, 1758) yolk-sac larvae (YSL) (17–30 dph). *Artemia* nauplii and gilthead seabream YSL were added twice per day at densities of 0.1–0.5 *Artemia* ml^{-1} and 300 gilthead seabream YSL per predator larvae. Rotifers were completed to five rotifers ml^{-1} twice per day. In addition, cultivated microalgae *Nannochloropsis gaditana* (Lubián, 1979) were added twice per day until 16 dph and then 0.8 g DW of a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Tokyo, Japan) per cubic metre twice per day until the end of the experiment.

2.3 | Oxygen consumption measurements

Larval oxygen consumption was measured by closed (static) respirometry, using a FireStingO_2 oxygen meter (PyroScience, Aachen, Germany). Pyroscience respiration vials (20 ml) with integrated optical oxygen sensors were used for larvae <8.8 mm and <1.53 mg (from 17 to 19 dph, n = 22), and common 100 ml Winkler bottles adapted with Pyroscience optodes were used for the bigger larvae with sizes <22.4 mm and <31.56 mg (from 17 to 30 dph, n = 95). Respiration chambers were volumetrically large relative to the size of the fish larvae (average 5000:1 and 13500:1 for the 20 and 100 ml chambers, respectively) as recommended by Peck and Moyano (2016). Sensors were calibrated before the start of each experiment at the experimental temperature in both oxygen-free water [0% O_2 saturation calibration, 30 g l^{-1} of sodium sulphite (Na_2SO_3)] and fully aerated water (100% O_2 sat calibration). A water-bath was used to maintain a stable temperature at 26 ± 0.1°C of variation, and walls were wrapped in green plastic paper to simulate similar ambient conditions as in the larval rearing tanks.

Each night before the RMR measurements, ABFT larvae were transferred to a new tank without prey for 8–9 h, allowing sufficient time for food to be cleared from the gut (Blanco *et al*., 2017) to minimize the effect of specific dynamic action. Larvae were individually collected from the rearing tank by using a big spoon and transferred to a bowl with filtered sea water (10 μm), using a red lantern in darkness to avoid larval disturbance. Then, they were transferred into a flat container with water saturated with oxygen (26°C) so that the larvae could enter the vial by itself completely submerging it. This method avoided any direct contact with the material used, and it was made sure the larvae were always submerged and in big spaces to diminish any handling-related stress. Respirometry measurements were conducted in light (RMR_{light}) and darkness ($RMR_{darkness}$). The temperature in the rearing tanks was similar to the experimental temperature. From 7:00 to 9:00 a.m., measures in dark conditions were carried out, and from 9:00 to 11:00 a.m. when the lights were switched on, measurements were performed in light conditions. During darkness measurement, the water-bath was completely covered to prevent any light entering from the outside. Chambers were filled with saturated filtered sea water (10 μm) at experimental temperature and overturned and separated from one another to avoid the larvae from seeing one another. Control measurements (no larvae) were run daily in light and darkness to control for bacterial and microbial respiration ("background respiration"). To avoid possible water stratification, a small piece of glass was introduced into the blank chambers and gently manually shaken a few times during the measure for water mixing (Rodgers *et al*., 2016). One to two drops of water from the larval water tanks were introduced into the blank chamber to compensate for the possible bacterial respiration associated with the larvae. The oxygen consumption rates in the controls were always <20% of those in chambers with a larva.

Once the larvae were introduced into the chambers, they were closed with a lid, and the measurement started. Oxygen consumption estimates (μmol O_2 l^{-1}) were performed on individual larvae and lasted between 30 and 55 min. Dark and light conditions were measured in different larvae. The first 10 min of the measurement was not considered to make sure the sensor was stable. Larval activity was visually checked at the beginning and at the end of each measurement and larvae showing abnormal swimming activity were discarded (*i.e*., continuous burst activity, collision to the walls) during darkness (using a red-light lantern) and light conditions, as suggested by Peck and Moyano (2016). Immediately after the oxygen measurement, larvae were euthanized using 50 mg l^{-1} clove oil (Guinama, Valencia, Spain), rinsed in distilled water, individually photographed for morphometric measures (standard length) and individually frozen in cryotubes at −80°C for later lyophilized DW (accurate to ±0.01 mg) and nucleic acid estimations. Under the binocular, individual larval stomach was visually inspected to ensure empty guts, and if any prey were found, the data from that larvae were excluded for further statistical analysis.

2.4 | Nutritional condition: nucleic acid analyses

Nucleic acids were analysed using a spectrofluorometer following the protocol by ICES (2004). Individual measures of RNA and DNA content were carried out using the whole larval body. Sodium lauryl sulphate was used to break cell membranes (plasmatic and nuclear) and release the nucleic acids and the fluorophore ethidium bromide (EB) to measure total nucleic acids. RNase was added to differentiate RNA from DNA. The difference between the total acid nucleic fluorescence and the DNA fluorescence (obtained by RNase) determines the RNA content, assuming insignificant residual fluorescence. RNA and DNA data were standardized as suggested by Caldarone *et al*. (2006), assuming a ratio of 2.4. DNA standard was constructed from genomic ultrapure calf thymus and RNA standard from baker yeast (Sigma-Aldrich, Madrid, Spain). The fluorescence was measured in runs of 20 samples to avoid the EB degradation. The analysis was completed within 4–5 months after sampling. For more details, see Blanco *et al*. (2019).

2.5 | Data analysis and statistics

The individual larval respiration (μmol O_2 individual^{-1} h^{-1}) was calculated as the decrease in oxygen concentration during the measurement time (slope), corrected with the blank and normalized by the chamber volume and larval DW to obtain the mass-specific oxygen consumption (μmol mg^{-1} h^{-1}). Larval-displaced volume was assumed to be negligible in the high respirometer-to-fish volume ratio. Larvae that died during handling were excluded from further analysis, as well as those that showed continuous burst activity or collisions with the chamber walls (*ca*. 6%). Once oxygen saturation reached 80%, the measurement was stopped, and the larva was removed from the chamber. A total of 117 larval measures were finally used for data analysis; 53 measurements were performed in light and 64 in darkness (Figure 1). Of those larvae, only 9 were in the flexion stage, whereas the rest, 108, were in the post-flexion stage (Kendall *et al*., 1984).

All statistical analyses were carried out using the R statistical software (www.r-project.org). A Kolmogorov–Smirnov test was used to assess normality, and homogeneity of variances was tested using Levene test. The mass-specific oxygen consumption (μmol mg^{-1} h^{-1}) and condition metric ratio (RNA/DNA and DNA/DW) data were log-transformed before statistical analyses. The effect of vial volume (20 and 100 ml) and larval stage (pre-flexion and post-flexion) during 17–20 dph was analysed using a three-way ANOVA test with treatment (light and darkness) also as a factor, in the oxygen consumption (μmol mg^{-1} h^{-1}) and condition metric ratios (RNA/DNA and DNA/DW). Consumption differences between larvae in the big (5000 l) and smaller (1500 l) tanks were also analysed using an ANOVA test. The significance level was accepted at $P < 0.05$.

A linear regression was performed on RMR (μmol O_2 individual^{-1} h^{-1}) and DW data on larvae in light and dark conditions. ANCOVA was run to test for differences in this RMR–DW relationship under light and darkness treatments.

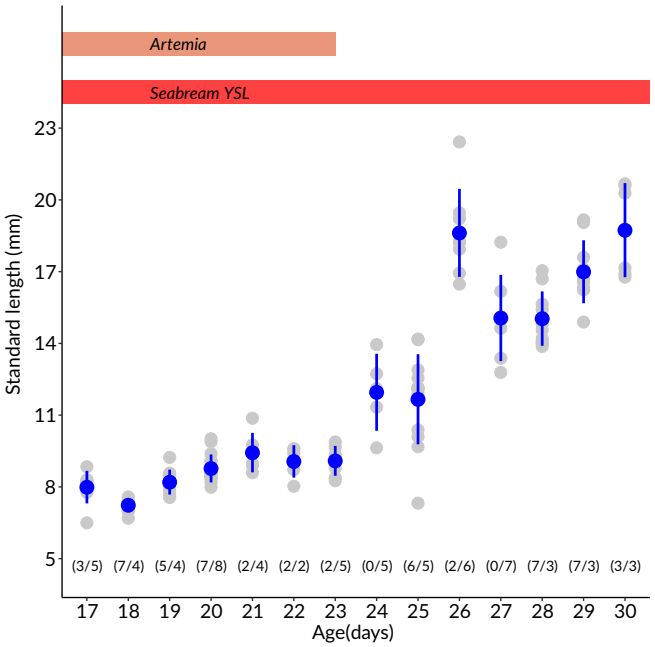

FIGURE 1 Diagram of the daily summary of the Atlantic bluefin tuna larvae used for oxygen consumption trials, including average (mean ± S.D.) standard length (mm), total number of larvae (*n*) sampled in light and dark conditions (*n* in light/*n* in darkness) and food source

Interindividual variations in the oxygen consumption and nutritional condition ratios were compared using the residuals of predictive regressions to explore the relationship without size effects. Residuals from the respiration–DW (respiration *vs*. DW) were compared to the RNA–DNA, DNA–DW and DW–DPH (size-at-age) residuals using linear regression. Condition metric residuals, RNA–DNA and DNA–DW were also compared to the DW–DPH (size-at-age) residuals by a linear regression.

3 | RESULTS

ABFT larvae displayed mass-specific growth rates of 32% per day and 20% per day from 17 to 26 dph (5000 l tank) and from 27 to 30 dph (1500 l), respectively. The size-at-length (DW–length relationship) increased exponentially with a slope of 3.3 (Figure 2; Table 1). RMR increased nearly isometrically with DW (b = 0.99, Figure 3; Table 1). The average mass-specific RMR was 0.424 ± 0.163 μmol O_2 mg^{-1} h^{-1} for 17–30 dph larvae. There were no significant differences in larval oxygen consumption due to respirometer size (20 *vs*. 100 ml) nor larval stage (pre- *vs*. post-flexion) (three-way ANOVA, $P > 0.05$). Similarly, there were no significant differences in larval oxygen consumption measured in light and darkness (Figure 3, ANCOVA, $P > 0.05$).

RNA/DNA increased allometrically with larval DW (b = 0.33, Figure 4a). DNA/DW decreased with increasing DW (Figure 4b). There were no significant differences in the RNA/DNA and in the DNA/DW between larvae maintained in light and darkness (ANCOVA, $P > 0.05$). Nutritional condition did not explain differences in size-at-age (DW–DPH) (not shown, ANCOVA, $P > 0.05$).

Interindividual differences in oxygen consumption were unrelated to nutritional conditions (RNA/DNA and DNA/DW) (Figure 5a,b, ANCOVA, $P > 0.05$). The size-at-age did not explain this variability either (Figure 5c, ANCOVA, $P > 0.05$).

4 | DISCUSSION

Obtaining metabolic rate estimates during the early life stages of fish can help identify key instants in energy demands and compartmentalization and improve the understanding of species biology and fisheries ecology, as well as optimize aquaculture success. To date, no study has ever measured oxygen consumption in ABFT larvae. This study explored how body size and light can affect the oxygen consumption of fish larvae during the piscivorous stage. The relationship between the metabolic rate and size showed a nearly isometric relationship from 0.60 to 31.56 mg, higher than the average relationship in other species, suggesting a high oxygen demand during the piscivorous stage, when the larvae metamorphose into the juvenile stage. No differences were found between light and darkness measurements, suggesting similar larval activity levels in both conditions. Nutritional condition (as RNA/DNA and DNA/DW) did not explain the interindividual differences in oxygen consumption, emphasizing the importance of the size as a major determinant of metabolic rate in this species.

4.1 | Larval growth

The daily specific growth rates in the present study were slightly lower than those reported in previous ABFT studies during the piscivorous stage due to the potential selection of the smaller larvae (Blanco *et al*., 2017; Reglero *et al*., 2014). The standard length *vs*. DW relationship estimated from laboratory-reared individuals was similar to relationships previously found in field-captured ABFT larvae (García *et al*., 2006).

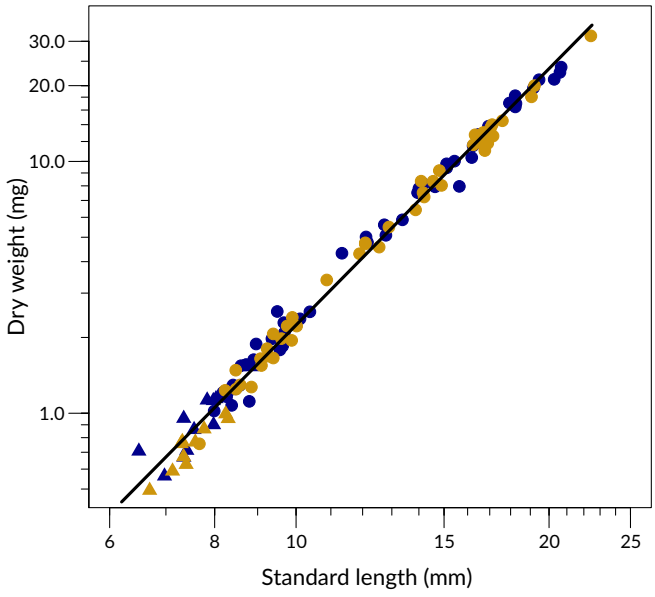

FIGURE 2 Standard length (mm) and dry weight (mg) relationship in bluefin tuna larvae reared at 26°C. Symbols are shape coded by size of the respiration chambers used (triangles, 20 ml; circles, 100 ml) and colour coded by light conditions during the respiration trial (yellow, light; blue, darkness). (▲) (●) Light (▲) (●) Darkness. See Table 1 for the regression equation

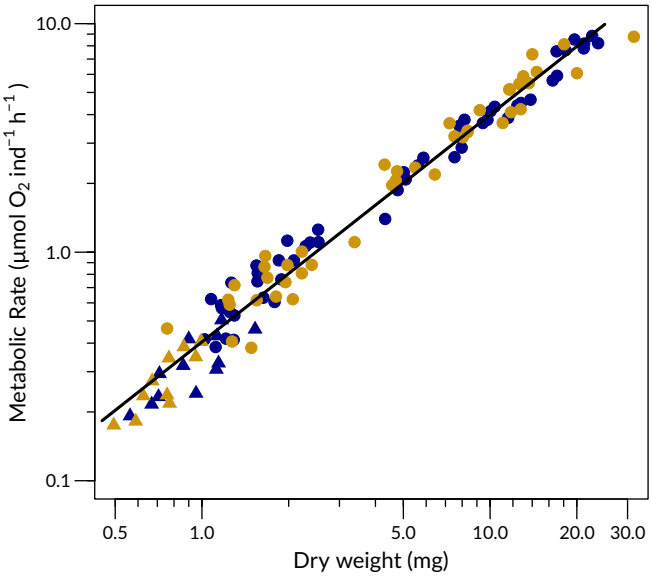

FIGURE 3 Routine metabolic rates (µmol O_2 individual^{-1} h^{-1}) and larval dry weight (mg) of Atlantic bluefin tuna larvae (17–30 days post-hatching) reared at 26°C. Symbols are shape coded by size of the respiration chambers used (triangles, 20 ml; circles, 100 ml) and colour coded by light conditions during the respiration trial (yellow, light; blue, darkness). No differences were found between measurements in light and darkness; therefore, only one linear regression is calculated. (▲) (●) Light (▲) (●) Darkness. See Table 1 for equation

TABLE 1 Allometric equations (±S.E.) of the standard length (SL) *vs*. dry weight (DW) relationship, routine metabolic rate (RMR, µmol individual^{-1} h^{-1}) *vs*. DW and nucleic acid ratios (RNA/DNA and DNA/DW) *vs*. DW for Atlantic bluefin tuna larvae, during the piscivorous phase from 17 to 30 days post-hatching at 26°C. Res.st.error: residuals standard error, *n*: number of measurements

Measurement	Equation	*b* (slope)	R^2	Res.st.error.	*n*
SL–DW	$DW = 0.001(\pm 1.076) \cdot SL^{3.382\ (\pm\ 0.030)}$	3.38	0.99	0.11	117
RMR–DW	$RMR = 0.404(\pm\ 1.025) \cdot DW^{0.994\ (\pm\ 0.015)}$	0.99	0.97	0.19	117
RNA/DNA–DW	$RNA/DNA = 1.699\ (\pm\ 1.048) \cdot DW^{0.332\ (\pm\ 0.029)}$	0.33	0.53	0.35	111
DNA/DW–DW	$DNA/DW = 22.309\ (\pm\ 1.024) \cdot DW^{-0.254\ (\pm\ 0.015)}$	−0.25	0.72	0.18	113

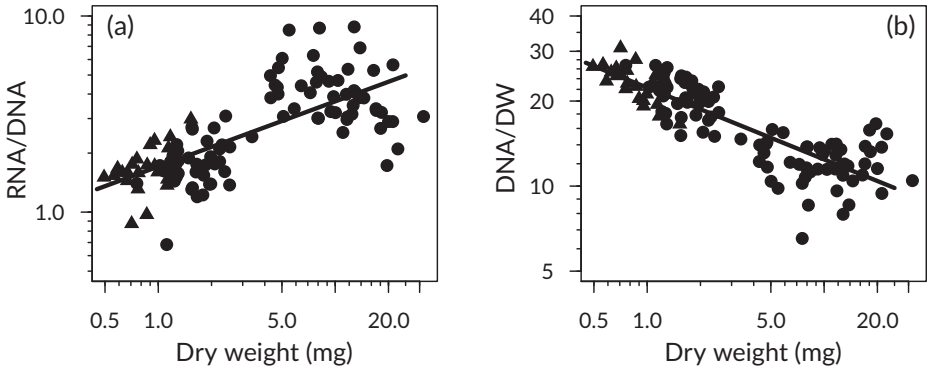

FIGURE 4 Relationship between Atlantic bluefin tuna larval nutritional condition as (a) RNA/DNA and (b) DNA/DW (dry weight) and individual DW (mg). Different symbols represent different sizes of the respiration chambers used (triangles, 20 ml; circles, 100 ml). No differences were found between light and darkness; thus, only the general linear regression is calculated. Regression equations are presented in Table 1

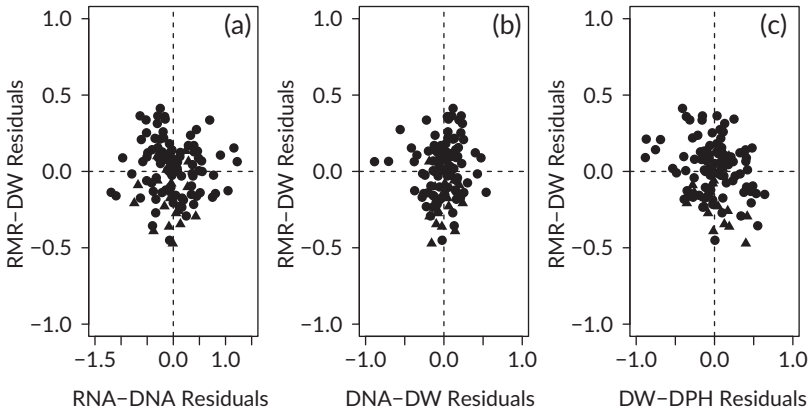

FIGURE 5 Relationship between the interindividual variability in routine metabolic rate (RMR; residuals of the relationship RMR–dry weight (DW), Figure 3) and several condition indices: (a) RNA-DNA, (b) DNA–DW and (c) DW-age (measures as days post-hatching, dph). Different symbols represent different sizes of the respiration chambers used (triangles, 20 ml; circles, 100 ml). No significant relationship was found between RMR–DW and any of the three condition indices

4.2 | Metabolic scaling with body size

The authors observed a nearly isometric metabolic exponent for ABFT piscivorous larvae at 26°C similar to that reported for piscivorous Pacific bluefin tuna larvae at 25°C (Miyashita *et al*., 1999). Miyashita *et al*. (1999) observed changes in the metabolic scaling exponent related to different ontogenetic stages that indicated that the highest oxygen consumption demand occurred before piscivory, probably related to the development of the digestive system and external characteristics that improve once piscivory started (Kaji, 2003; Yúfera *et al*., 2014). In the related species Atlantic mackerel *Scomber scombrus* (Linnaeus, 1758), Giguere *et al*. (1988) also reported an isometric exponent with increasing body mass during the larval stage.

In the present study, an average of 0.410 μmol mg^{-1} h^{-1} of oxygen was consumed by piscivorous 0.60–31.56 mg DW ABFT larvae. This consumption is within the range measured manometrically in smaller Pacific mackerel *Scomber japonicus* (Houttuyn, 1782) larvae (Hunter & Kimbrell, 1980) and bay anchovy *Anchoa mitchilli* (Valenciennes, 1848) using a polarographic oxygen electrode (Houde & Schekter, 1983). Due to methodological differences, it is difficult to compare the mass-specific oxygen consumption rates with those larvae of other non-scombrid species. Nonetheless, the high scaling component (b = 0.99) along with the high intercept (a = 0.4) or the rate at any given size obtained in this study suggests that, at least during the piscivorous stage, ABFT larval oxygen consumption is larger than in other fish larvae (Peck & Moyano, 2016). During this piscivorous stage, growth rates can reach 54% per day in comparison with the rates of 35% per day during the previous planktivorous stage (Reglero *et al*., 2014; Blanco *et al*., 2017, 2019). The need to maintain these high growth and developmental rates during the piscivorous stage is likely the result of the high oxygen consumption in ABFT larvae.

Apart from the ontogenetic changes in oxygen consumption studied in Pacific bluefin tuna larvae (Miyashita *et al*., 1999), and the optimal oxygen ranges described for yellowfin tuna *Thunnus albacares* (Bonnaterre, 1788) larvae (Wexler *et al*., 2011), the rest of the oxygen-related studies in scombrids have been mainly done in tuna adults. In adults, the relationship between fish size and SMR suggests an allometric relationship with b = 0.5–0.6 (*e.g*., Brill, 1979, 1987; Dewar *et al*., 1994a), and for RMR it is 1.18–1.19 (*e.g*., Gooding *et al*., 1981; Graham & Laurs, 1982). Tuna adults appear to have a lower standard metabolic exponent compared with the general exponent of 0.8 proposed in the juvenile and adult stages of fishes (Clarke & Johnston, 1999; Korsmeyer & Dewar, 2001; Wieser, 1995). This study can only speculate the SMR changes in ontogeny in ABFT, as its attempt to measure SMR as $RMR_{darkness}$ was unsuccessful. Nonetheless, those results in adult tuna individuals suggest that mass-specific SMR decreases relatively rapidly as body mass increases (Brill, 1987). The decrease in the SMR might be explained by a decrease in the growth and maintenance rates. The high routine metabolic rate of adults

compared with the general trend might be mainly contributed by the cost of endothermy and the extreme swimming capability which result in greater locomotion cost (ram-ventilation and swim power) in comparison with other fishes (Graham & Dickson, 2004). Adult bluefin tuna demand a higher oxygen concentration than other marine fishes (Korsmeyer & Dewar, 2001).

4.3 | Routine metabolic rate in light and darkness

The estimates of minimum metabolic demands, SMR, in ectotherms require that the organism is "in a post-absorptive, calm, inactive state after proper thermal acclimation" (Chabot *et al*., 2016a). These conditions would require long starvation, acclimation and measuring times (≥24 h) in adults, which can be especially difficult to meet in early stages of fish. Therefore, three common methods have been used in young larvae to obtain SMR estimates. First, anaesthetics can be used to induce a resting state, and this has been the most common method used so far in fish larvae (De Silva *et al*., 1986; Kiorbøe *et al*., 1987; Moyano *et al*., 2014) and adult individuals (Brill, 1979, 1987). Nonetheless, their use is not recommended because they may induce metabolism depression, and their effect may differ across anaesthetic types (Javahery *et al*., 2012; Peck & Moyano, 2016). In general, anaesthetized larvae consumed 30% of the oxygen consumed by non-anaesthetized larvae (*e.g*., Davenport & Lonning, 1980; De Silva *et al*., 1986; Houlihan *et al*., 1995). Second, SMR can be estimated from extrapolating curves of oxygen consumption and swimming speed back to zero velocity (Dewar *et al*., 1994b; Gooding *et al*., 1981; Graham & Laurs, 1982). Third, other studies use darkness to reduce activity, as most larval fish are visual predators (Ruzicka & Gallager, 2006a; Yamashita & Bailey, 1989). The cost of routine movements has been estimated to be 1.5 times higher than that of darkness (or the cost of activity, in light being 70%–80% of the total metabolic rate) (Peck & Moyano, 2016; Porter, 2001; Ruzicka & Gallager, 2006a), a factor that is slightly higher than the RMR:SMR ratio, when the definition of SMR is fulfilled. Pre-trials indicated that neither anaesthetics nor measurements of active metabolisms are an option for the delicate ABFT larvae (pers. obs., see the "Methodological challenges" section). Therefore, this study tested whether $RMR_{darkness}$ would be lower than RMR_{light} and potentially be used as proxy for SMR.

The results showed similar RMR in light and darkness, suggesting similar larval activity levels under both conditions. ABFT larvae significantly reduced their swimming activity in the respiration chambers in comparison to the high activity they generally have in the rearing tanks, but still, they did display some level of activity (pers. obs.). This reduced swimming activity within a respirometer relative to freely swimming larvae was expected (Ruzicka & Gallager, 2006a). These authors compared the swimming behaviour of larval cod in large tanks (250 l) and in respiration chambers (6.5 ml) and found that the activity levels in the tanks were about ×3.8 (×4.9) higher in the absence (presence) of prey compared to that inside the small respirometers. Therefore, similar RMR_{light} and $RMR_{darkness}$ might be the consequence of a continued activity of the larvae in darkness or of a diminished activity in light. One can also speculate that under light conditions but in the absence of prey, ABFT larvae might have changed their behaviour to balance the activity consumption with the energy needed to grow and survive (Wieser *et al*., 1988).

Other aspects that could explain the similarity in RMR_{light} and $RMR_{darkness}$ of ABFT larvae may be related to the effects of hunger and/or potential handling stress. First, hunger is known to suppress larval swimming activity and behaviour in Atlantic cod *Gadus morhua* (Linnaeus, 1758) and Atlantic herring *Clupea harengus* (Linnaeus, 1758) larval species, although generally at longer time scales (days, not hours) (Illing *et al*., 2018; Ruzicka & Gallager, 2006b). In this study, to ensure larval fasting, larvae were transferred to a new tank without prey each night before the RMR measurements. In that tank, lights were switched on as usual (9:00 a.m.), but prey were absent in the tanks. RMR_{light} estimates were performed up to 2 h after the usual start of larval feeding (from 9:00 to 11:00 a.m.). After a couple of hours without food, larvae may have decreased foraging activity (and potentially other metabolic demands) to balance the activity consumption with the energy needed to grow (Wieser *et al*., 1988).

Second, the method applied to assess metabolism in this study (closed respirometry) uses single measurements of each individual larva and does not allow long-term acclimation times; thus, the effect of handling stress cannot be excluded. Handling stress is also a known issue in ABFT larvae, as they are extremely sensitive to any type of manipulation (De la Gándara *et al*., 2010). To minimize the effect of handling stress, the larvae were loaded in the chambers using a careful protocol in which they are allowed to enter the chambers by themselves. Still, this effect cannot be completely excluded and may have masked any treatment differences. Intermittent-flow respirometry would be a better alternative to closed respirometry in this context, as it allows successive measurements in the same individual over several hours, minimizing the handling effect. Unfortunately, this method is technically challenging when used with fast-growing fish larvae and especially in tuna and bonito larvae (see the "Methodological challenges" section). In fact, only three studies have used it with fish larvae in the past decade (McKenzie *et al*., 2008; McLeod *et al*., 2013; McLeod & Clark, 2016), although this trend is increasing due to quick technological developments.

4.4 | The link between nutritional condition and metabolism

A positive correlation was found between RNA/DNA and larval DW, which suggests that the larvae used in the RMR measurements were in good nutritional condition. High interindividual variability in size-at-age (DW–DPH), RNA/DNA and DNA/DW was observed in the larvae used during this experiment. The standard errors of all three variables were higher than 20%. Such high variability in nutritional condition is common in fish larvae and has been related to ontogenetic changes (Foley *et al*., 2016; Meyer *et al*., 2012). In addition, as seen in previous studies, RNA/DNA might not be the most representative nutritional condition

estimate in ABFT larvae due to its high variability, and DNA/DW is considered more stable (Bergeron, 1997; Blanco *et al*., 2017).

Nutritional condition (RNA/DNA, DNA/DW) did not explain the differences in interindividual variability in RMR. These results are in accordance with those of other species, like Atlantic herring, where it was also found that the differences in SMR were not explained by the RNA/DNA (Moyano *et al*., 2018). Also, this study's results show that larval size-at-age (DW–DPH) did not explain the differences in interindividual variability. These results emphasize that larval size is the major explanatory variable explaining changes in RMR of ABFT larvae, and neither growth rate nor nutritional condition is related with interindividual differences in RMR under *ad libitum* conditions.

4.5 | Methodological challenges

The lack of information about metabolic costs in scombrid larvae is probably related to the challenges associated to larval handling before and during respirometry trials. Scombrids, and in particular ABFT, are very sensitive during the larval stage. The larvae need to be handled by experienced people, and still, handling-related mortality occurs as soon as larvae are sampled from the rearing tanks. These larvae are very sensitive to any contact with container walls (*e.g*., beakers), transferring devices (*e.g*., pipette) and air exposure, making experimental tests very challenging.

The choice of respirometer type is not trivial in experiments with scombrid larvae. ABFT larvae show spontaneous movements, and compared to other species (*e.g*., Atlantic herring, Illing *et al*., 2018), pause or resting behaviour is infrequent, especially in big larvae. This study selected bigger chamber sizes than those recommended for other larval fish, suggesting volume chamber (ml):wet weight (g) ratios of 100:1–300:1 (Peck & Moyano, 2016, assuming DW as 20% of the wet weight). In the 20 ml chamber, ranges of 2500:1 in the biggest larvae to 8000:1 in the smallest larvae were used and in the 100 ml chamber 850:1 and 25000:1, respectively. Previous tests by the authors with 4 ml chambers (PyroScience) in smaller ABFT larvae (0.10–0.36 mg DW) also led to higher ratios than those recommended (2000:1–8000:1). Surprisingly, those tests showed relatively low handling mortality (<1%), so they may be a good alternative for smaller stages. In summary, there needs to be a compromise between the chamber volume to fish size and the volume needs of these sensitive larvae to avoid much contact with the respirometer walls, but it is believed that 4, 20 and 100 ml respirometers are adequate for 0.1–0.4, 0.5–1.5 and 0.60–31.56 mg DW scombrid larvae.

Intermittent-flow-through respirometry is considered a more appropriate method to measure the metabolic rates in fish than close respirometry, because oxygen consumption is recorded during long periods of time allowing for some initial acclimation to the system (Chabot *et al*., 2016a; Chabot *et al*., 2016b; Peck & Moyano, 2016). Nonetheless, this method is still very challenging for delicate early life stages and has only seldom been used (McKenzie *et al*., 2008; McLeod *et al*., 2013; McLeod & Clark, 2016). Attempts at using intermittent-flow respirometers (mini swim tunnel respirometer, Loligo Systems, Viborg, Denmark) with post-flexion ABFT larvae have been unsuccessful. Larvae were not able to overcome the selected minimum flow and maintain a specific swimming speed. Instead, they turn around to avoid the flow and hit themselves against the walls, which caused the mortality of 100% of the tested larvae. Further research (and likely technological development) is therefore needed to obtain reliable estimates of the metabolic rates of scombrid larvae at various levels of activity. This information is essential to quantify changes in metabolic rates during ontogeny to ontogenetic thresholds in energy demands and compartmentalization to improve understanding of the species.

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