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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 these 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 purposes. This study for the first time: i) estimates the relationship between routine metabolic rate and larval dry weight (mass scaling exponent) at a constant temperature of 26 °C, ii) measures routine metabolic rate under light and darkness, and iii) tests whether the inter-individual differences in routine metabolic rate are related to larval nutritional status (RNA/DNA and DNA/DW). Routine metabolic rate scaled nearly isometrically with body size (b=0.99, 0.60 to 31.56 mg dry weight), in contrast to the allometric relationship observed in most fish larvae (average b=0.87). Our results show no significant differences in larval routine metabolic rate under light and darkness, suggesting similar larval activity levels in both conditions. Size explained most of the variability in routine metabolic rate (97%), and nutritional condition was unrelated to the inter-individual differences in routine metabolism. This study first reports metabolic rates of Atlantic bluefin tuna larvae and discusses the challenges of performing bioenergetic studies with early life stages of scombrids.

Key words: Atlantic bluefin tuna, larvae, metabolism, respiration, RMR, nutritional condition

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INTRODUCTION

Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus, 1758) is one of the most valuable pelagic resources worldwide (Juan-Jordá et al., 2013). Despite 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 on the physiology of these early life stages can help identify vulnerabilities during ontogeny and increase larval survival. For example, increases in the 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 improving larviculture protocols by optimizing necessary daily nutritional requirements. Besides, translating larval physiological knowledge into predictive models, might improve our understanding of species response to perturbations and develop more reliable indices of annual recruitment (Peck et al., 2013; Jørgensen et al., 2016; Reglero et al., 2018a, 2018b)

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 survive, grow and reproduce (Post & Lee, 1996). Metabolic rate (M) changes with body size (B), following the equation $M = a B^b$. 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 b-slope higher than 1 during the pre-metamorphic stages decreasing to 0.8 in later life stages (Killen et al., 2007). Quantifying

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the changes in metabolic rates during ontogeny can help us identify key changes in energy demands and compartmentalization.

Measurements of metabolic rates in early life stages of marine fish are still relatively scarce compared to 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 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 early life stages of warm water species, such as scombrids, is especially relevant as they need to support growth rates exceeding 30% day^{-1} (Houde, 1989; McCarthy & Fuiman, 2011). To our 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 have already developed 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 doing respirometry tests in confinement. One solution to reduce this confinement stress, not yet tested in larval stages, is running the respirometry measurements in darkness. Since these larvae, as most marine larvae, are visual feeders (Margulies, 1997), one expects that they

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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). In this study, we used two different indices to determine larval nutritional condition: the RNA/DNA and the DNA/DW. 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 RNA is directly proportional to the protein synthesized in the cell which is highly dependent to 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 other hand, the DNA/DW show larval cell condition, increasing when condition decreases, because less weight is attributed per cell number (Bergeron, 1997).

The present study reports the first estimates of routine metabolic rates in Atlantic bluefin tuna larvae. The main objectives of our study are: i) estimate the relationship between routine metabolic rate and the larval dry weight (DW) (mass scaling exponent) at 26 °C, ii) measure routine metabolic rate in light and darkness, and iii) tests whether the inter-individual differences in routine metabolic rate 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.

MATERIALS AND METHODS

ETHICAL STANDARDS

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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).

LARVAL REARING

Fertilized Atlantic bluefin tuna (ABFT) eggs were obtained from naturally spawning captive adults in the farming facilities at El Gorguel, Cartagena (SE Spain), owned by Caladeros del Mediterráneo S.L. The eggs were collected and transported to the Spanish Institute of Oceanography (IEO) facilities in Mazarrón (SE 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 L^{-1}), where the larvae hatched and remained until 26 days post hatch (dph). At 26 dph all the larvae were removed from the tank (n=203), and 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 to 26 dph) or from the 1500 L tank (27 to 30 dph). During the larval rearing period, the photoperiod regime was 14 hours of light and 10 hours of darkness, 14L:10D. Rearing was conducted at ambient temperature (24 to 26 °C) and a salinity of 38. Preys were always *ad libitum* in the tanks and the feeding schedule consisted of i) enriched rotifers *Brachionus plicatilis* (Müller, 1786) (4-16 dph), ii) enriched *Artemia* nauplii *Artemia franciscana* instar II (Kellogg, 1906) (AF, INVE AQUACULTURE, Belgium) (11-23 dph), and iii) gilthead sea bream *Sparus aurata* (Linnaeus, 1758) yolk-sac larvae (YSL) (17-30 dph). *Artemia* nauplii and gilthead sea bream YSL were added twice per day at densities of 0.1-0.5 *Artemia* mL and 300 gilthead sea bream YSL per predator larvae. Rotifers were completed to 5 rotifers mL^{-1} twice per day. In addition, cultivated microalgae *Nannochloropsis gaditana* (Lubián, 1979) were added two times per day until 16 dph, and then 0.8 g dry weight of paste of concentrated

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Chlorella (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) per m^3 three times a day until the end of the experiment.

OXYGEN CONSUMPTION MEASUREMENTS

Larval oxygen consumption was measured by closed (static) respirometry, using a FireStingO_2 oxygen meter (PyroScience, Germany). Pyroscience respiration vials of 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 both in oxygen free water (0% O_2 sat 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 in order to maintain 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 during 8-9 hours, allowing sufficient time for food to be cleared from the gut (Blanco et al., 2017) to minimize the effect of specific dynamic action (SDA). Larvae were individually collected from the rearing tank by using a big spoon and transferred to a bowl with filtered sea water (10µm), using a red lantern in darkness in order to avoid larval disturbance. Then, they were transferred into a flat container with water saturated with oxygen (26c) so that the larvae could enter the vial by itself completely submerging it. In this way, we avoided any direct contact with the material used and we made sure the larvae were always submerged and in big spaces in order to diminish any handling related stress. Respirometry measurements were conducted in light (RMR_{light}) and darkness ($RMR_{darkness}$). Temperature in the rearing tanks was similar to the experimental temperature. From 7:00–9:00 in the morning, measures in darkness conditions were

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carried out and from 9:00–11:00 when the lights were switch on, measures were done 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 seawater (10 µm) at experimental temperature and overturned and separated from each other to avoid the larvae to see each other. Control measurements (no larvae) were run daily in light and darkness to control for bacterial and microbial respiration ("back-ground respiration"). In order to avoid possible water stratification, a small piece of glass was introduced to the blank chambers, and gently manually shaken few times during the measure for water mixing (Rodgers et al., 2016). One to two drops of water from the larval water tanks was introduced to the blank chamber in order to compensate for the possible bacterial respiration associated with the larvae. Oxygen consumption rates in the controls were always <20% of those in chambers with a larva.

Once the larvae were introduced in the chambers, they were closed with a lid and the measurement started. Oxygen consumption estimates (µmol O_2 L^{-1}) were done on individual larvae and lasted between 30 and 55 minutes. Dark and light conditions were measured in different larvae. The first 10 minutes of the measurement were removed to make sure the sensor was stable. Larval activity was visually checked at the beginning and at the end of each measurement and discard larvae showing abnormal larval swimming activity (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© Spain), rinsed in distilled water, individually photographed for morphometric measures (standard length) and individually frozen in cryotubes at -80 °C for later lyophilized dry weight (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.

NUTRITIONAL CONDITION: NUCLEIC ACID ANALYSES

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Nucleic acids were analyzed using a spectrofluorometer followed the protocol by ICES (2004). Individual measures of RNA and DNA content were carried out using the whole larval body. Sodium lauryl sulfate (SDS) 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) determine 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 (Sigma Aldrich) and RNA standard from baker yeast (Sigma Aldrich). The fluorescence was measured in runs of 20 samples in order to avoid the EB degradation. The analysis was completed within 4–5 months after sampling. For more details, see Blanco et al. (2019).

DATA ANALYSIS AND STATISTICS

Individual larval respiration (µmol O_2 individual^{-1} h^{-1}) was calculated as the decline in oxygen concentration during the measurement time (slope), corrected with the blank and normalized by the chamber volume and larval dry weight 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 were excluded from further analysis when died during handling, showed continuous burst activity or collisions with the chamber walls were observed (~ 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 the data analysis. 53 measurements were done in light and 64 in darkness (Fig. 1). Of those larvae, only 9 were in flexion stage, while the rest 108, were in post-flexion (Kendall et al., 1981).

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. Mass-specific oxygen consumption (µmol mg^{-}

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^{1} h^{-1}) and condition metric ratios (RNA/DNA and DNA/DW) data were log-transformed prior to statistical analyses. The effect of vial volume (20 mL and 100 mL) and larval stage (pre-flexion and in post-flexion) during 17 to 20 dph was analyzed by a three-way ANOVA with treatment (light and darkness) also as 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) tank was also analyzed by an ANOVA. 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 in darkness. Analysis of covariance (ANCOVA) was run to test for differences in this RMR-DW relationship under light and darkness treatments.

Inter-individual variations in the oxygen consumption and nutritional condition ratios were compared using residuals of predictive regressions in order 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 metrics residuals, RNA-DNA and DNA-DW were also compared to the DW-DPH (size-at-age) residuals by a linear regression.

RESULTS

ABFT larvae displayed mass-specific growth rates of 32% d^{-1} and 20% d^{-1} from 17 to 26 dph (5000 L tank) and from 27 to 30 dph (1500 L), respectively. Size-at-length (DW-length relationship) increased exponentially with a slope of 3.3 (Fig. 2, Table 1). RMR increased near isometrically with DW (b=0.99, Fig. 3, Table 1). Average mass-specific RMR was 0.424 ± 0.163 µmol O_2 mg^{-1} h^{-1} for 17 to 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 (Fig. 3, ANCOVA, $p>0.05$).

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The RNA/DNA increased allometrically with larval DW (b=0.33, Fig. 4a). The DNA/DW decreased with increasing DW (Fig. 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, $p>0.05$).

Inter-individual differences in oxygen consumption were unrelated to nutritional condition (RNA/DNA and DNA/DW) (Fig. 5a, b, ANCOVA, $p>0.05$). Size-at-age did not explain this variability either (Fig. 5c, ANCOVA $p>0.05$).

DISCUSSION

Obtaining metabolic rate estimates during the early life stages of fish can help us identify key instants in energy demands and compartmentalization and improve our understanding of the species biology and fisheries ecology, as well as optimize aquaculture success. To date, no study has ever measured oxygen consumption in ABFT larvae. Here we 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 near 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 metamorphoses 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 inter-individual differences in oxygen consumption, emphasizing the importance of the size as a major determinant of metabolic rate in this species.

LARVAL GROWTH

Daily specific growth rates in our study were slightly lower than those reported in previous ABFT studies during the piscivorous stage due to the potential selection of the smaller larvae (Reglero et al., 2014; Blanco et al., 2017). Our standard length *vs.* DW

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relationship estimated from laboratory-reared individuals was similar to relationships previously found in field-captured ABFT larvae (García et al., 2006).

METABOLIC SCALING WITH BODY SIZE

We observed a near isometric metabolic exponent for ABFT piscivorous larvae at 26ºC similar to that reported for piscivorous Pacific bluefin tuna larvae at 25 ºC (Miyashita et al., 1999). Miyashita et al. (1999) observed changes in the metabolic scaling exponent related to different ontogenetic stages that indicated the highest oxygen consumption demand occurred before piscivory, probably related to the development of the digestive system and external characteristics that improve once they start piscivory (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 our study, an average of 0.410 µmol mg^{-1} h^{-1} of oxygen was consumed by piscivorous 0.60 to 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 our mass-specific oxygen consumption rates with those larvae of other non-scombrid species. However, the high scaling component (b=0.99) along with the high intercept (a=0.4) or the rate at any given size obtained in our 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% d^{-1} in comparison with rates of 35% d^{-1} during the previous planktivourous 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.

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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 suggest an allometric relationship with b=0.5–0.6 (e.g., Brill, 1979, 1987; Dewar & Graham, 1994) and for RMR it is 1.18–1.19 (e.g., Gooding et al., 1981; Graham & Laurs, 1982). Tuna adults appear to have lower standard metabolic exponent compared with the general exponent of 0.8 proposed in juvenile and adult stages of fishes (Wieser, 1995; Clarke & Johnston, 1999; Korsmeyer & Dewar, 2001). We can only speculate about the SMR changes in ontogeny in ABFT, as our attempt to measure SMR as $RMR_{darkness}$ was unsuccessful. However, 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 scaling 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 that other marine fishes (Korsmeyer & Dewar, 2001).

ROUTINE METABOLIC RATE IN LIGHT AND DARKNESS

Estimates of minimum metabolic demands, SMR, in ectotherms require that the organisms is “in a post-absorptive, calm, inactive state after proper thermal acclimation” (Chabot et al., 2016b). These conditions would require long starvation conditions, acclimation and measuring times (≥ 24h) 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 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).

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However, 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, anesthetized 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 (Gooding et al., 1981; Graham & Laurs, 1982; Dewar et al., 1994b). Third, other studies use darkness to reduce activity, as most larval fish are visual predators (Yamashita & Bailey, 1989; Ruzicka & Gallager, 2006a). The cost of routine movements has been suggested to be 1.5 times higher than those of darkness (or the cost of activity, in light being 70–80% of the total metabolic rate) (Porter, 2001; Ruzicka & Gallager, 2006a; Peck & Moyano, 2016), 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 (personal observation, see Methodological challenges section). Therefore, in this study we tested whether $RMR_{darkness}$ would be lower than RMR_{light} and potentially be used as proxy for SMR.

Our 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 (personal observation). This reduced sswimming activity within a respirometer relative to freely swimming larvae was expected (Ruzicka & Gallager, 2006a). These authors compared swimming behaviour of larval cod in large tanks (250 L) and in respiration chambers (6.5 mL), and found that activity levels in the tanks were about 3.8x (4.9x) higher in absence (presence) of prey compared to that inside the small respirometers. Therefore, our similar RMR_{light} and $RMR_{darkness}$ might be consequence of a continued activity of the larvae in darkness or of a diminishment of the activity in light. One can also speculate that under light conditions but in absence of prey, ABFT larvae might have changed their behavior in order to

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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 behavior 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) (Ruzicka & Gallager, 2006b; Illing et al., 2018). In this study, in order to ensure larval fasting, larvae were transferred to a new tank without prey each night before the RMR measurements. In that tank, lights where switched on as usual (9:00 am), but prey was absent from the tanks. RMR_{light} estimates were done up to two hours after the usual start of larval feeding (from 9:00 to 11:00 am). After a couple of hours without food, larvae may have decreased foraging activity (and potentially other metabolic demands) in order to balance the activity consumption with the energy needed to grow (Wieser et al., 1988).

Second, the methodology 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). In order to minimize the effect of handling stress, we loaded the larvae 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 handling effect. Unfortunately, this methodology is technically challenging when used with fast-growing fish larvae, and especially in tuna and bonito larvae (see Methodological challenges section). In fact, only three studies have used it with fish larvae in the last decade

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(McKenzie et al., 2008; McLeod et al., 2013; McLeod & Clark, 2016), although this trend is increasing due to quick technological developments.

THE LINK BETWEEN NUTRITIONAL CONDITION AND METABOLISM

We found a positive correlation between RNA/DNA and larval DW, which suggests that the larvae used in the RMR measurements were in good nutritional condition. High inter-individual variability in size-at-age (DW-DPH), RNA/DNA and DNA/DW was observed in the larvae used during this experiment. 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 (Meyer et al., 2012; Foley et al., 2016). Besides, 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., 2019).

Nutritional condition (RNA/DNA, DNA/DW) did not explain the differences in inter-individual variability in RMR. These results are in accordance with those of other species, like Atlantic herring, where they also found that the differences in SMR were not explained by the RNA/DNA (Moyano et al., 2018). Also, our results show that larval size-at-age (DW-DPH) did not explain the differences in inter-individual 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 inter-individual differences in RMR under *ad libitum* conditions.

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. Larval handling needs to be done by experienced people, and still, handling-related mortality occurs as soon as larvae are sampled from the rearing tanks. These larvae are very

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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. In this study, we selected bigger chamber sizes than those recommended for other larval fish, suggesting volume chamber (ml):wet weight (gr) ratios of 100:1-300:1 (Peck & Moyano, 2016, assuming DW as the 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 to 0.36 mg DW) also led to higher ratios than those recommended (2000:1 to 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 to the respirometer walls, but we believe 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 methodology to measure 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., 2016b, 2016a; Peck & Moyano, 2016). However 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). Our attempts of using intermittent-flow respirometers (mini swim tunnel respirometer, Loligo® System) 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 in order to avoid the flow and hit themselves towards the walls, which caused the mortality of 100% of the tested larvae. Further research (and likely technological development) is

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therefore needed to obtain reliable estimates of metabolic rates of scombrid larvae at various levels of activity. This information is essential to quantify changes in metabolic rates during ontogeny in order to ontogenetic thresholds in energy demands and compartmentalization to improve our understanding of the species.

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Table 1. Allometric equations (± SE) of the standard length (SL) *vs*. dry weight (DW) relationship, routine metabolic rate (RMR, µmol ind^{-1} h^{-1}) *vs*. dry weight, and nucleic acid ratios (RNA/DNA and DNA/DW) *vs*. dry weight for Atlantic bluefin tuna larva, during the piscivorous phase from 17 to 30 dph at 26 °C.

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

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1 **FIGURE CAPTIONS**

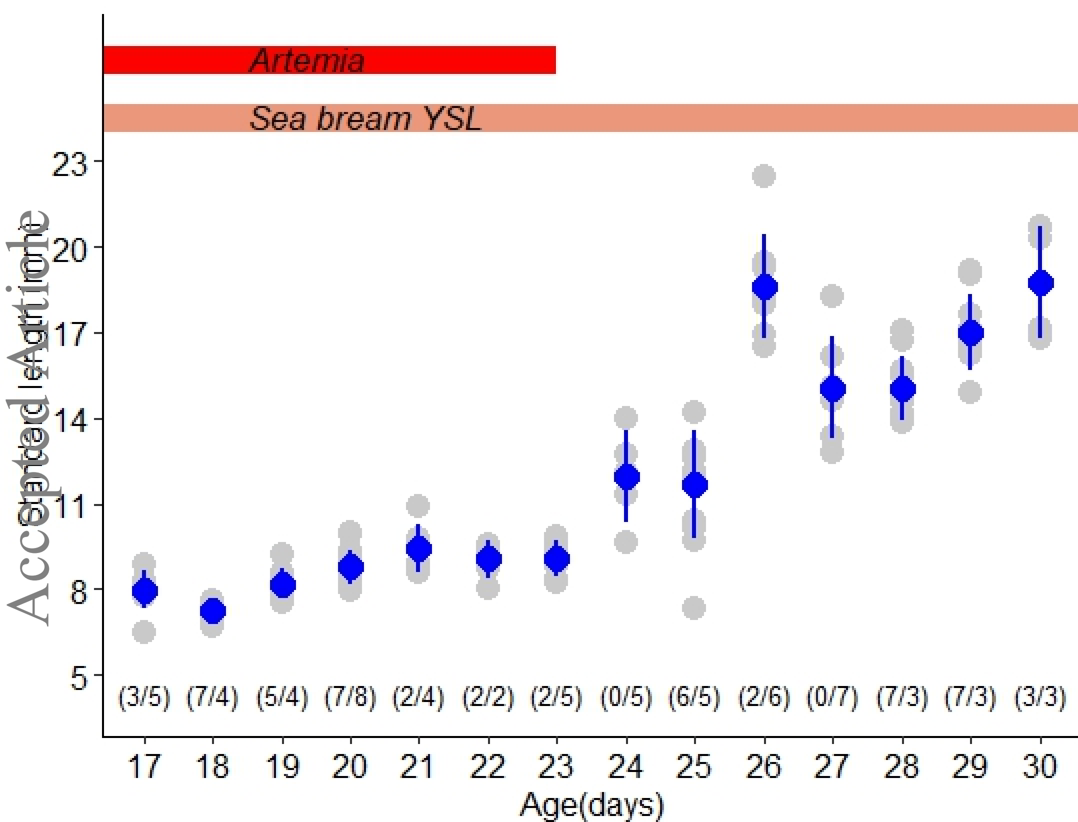
2 Figure 1. Diagram of the daily summary of the Atlantic bluefin tuna larvae used for
3 oxygen consumption trials, including average (mean ± SD) standard length (mm) total
4 number of larvae (n) sampled in light and dark conditions (n in light/n in dark), and food
5 source.

6 Figure 2. Standard length (mm) and dry weight (mg) relationship in bluefin tuna larvae
7 reared at 26°C. Symbols are shape-coded by size of the respiration chambers used
8 (triangles, 20mL, circles, 100mL) and color-coded by light conditions during the
9 respiration trial (yellow, light, blue, darkness). See Table 1 for the regression equation.

10 Figure 3. Routine metabolic rates (µmol O_2 individual^{-1} h^{-1}) and larval dry weight (mg)
11 of Atlantic bluefin tuna larva (17 to 30 days post hatch) reared at 26°C. Symbols are
12 shape-coded by size of the respiration chambers used (triangles, 20mL, circles, 100mL)
13 and color-coded by light conditions during the respiration trial (yellow, light, blue,
14 darkness). No differences were found between measurements in light and darkness,
15 therefore only one linear regression is calculated. See Table 1 for equation.

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

21 Figure 5. Relationship between the inter-individual variability in routine metabolic rate
22 (RMR; residuals of the relationship RMR-dry weight, Fig. 3) and several condition
23 indices: a) RNA/DNA, b) DNA-DW, and c) DW-Age (measures as days post-hatch,
24 DPH). Different symbols represent different size of the respiration chambers used
25 (triangles, 20mL, circles, 100mL). No significant relationship was found between RMR-
26 DW and any of the three condition indices.



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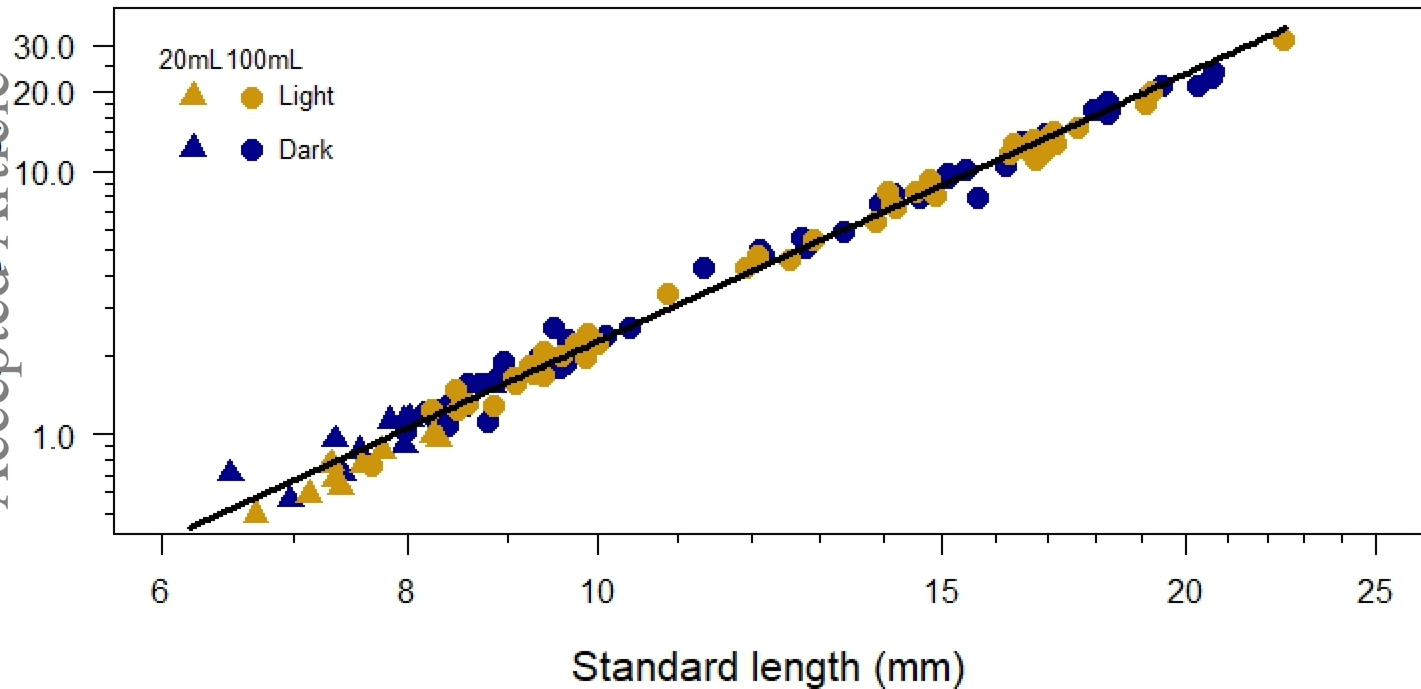

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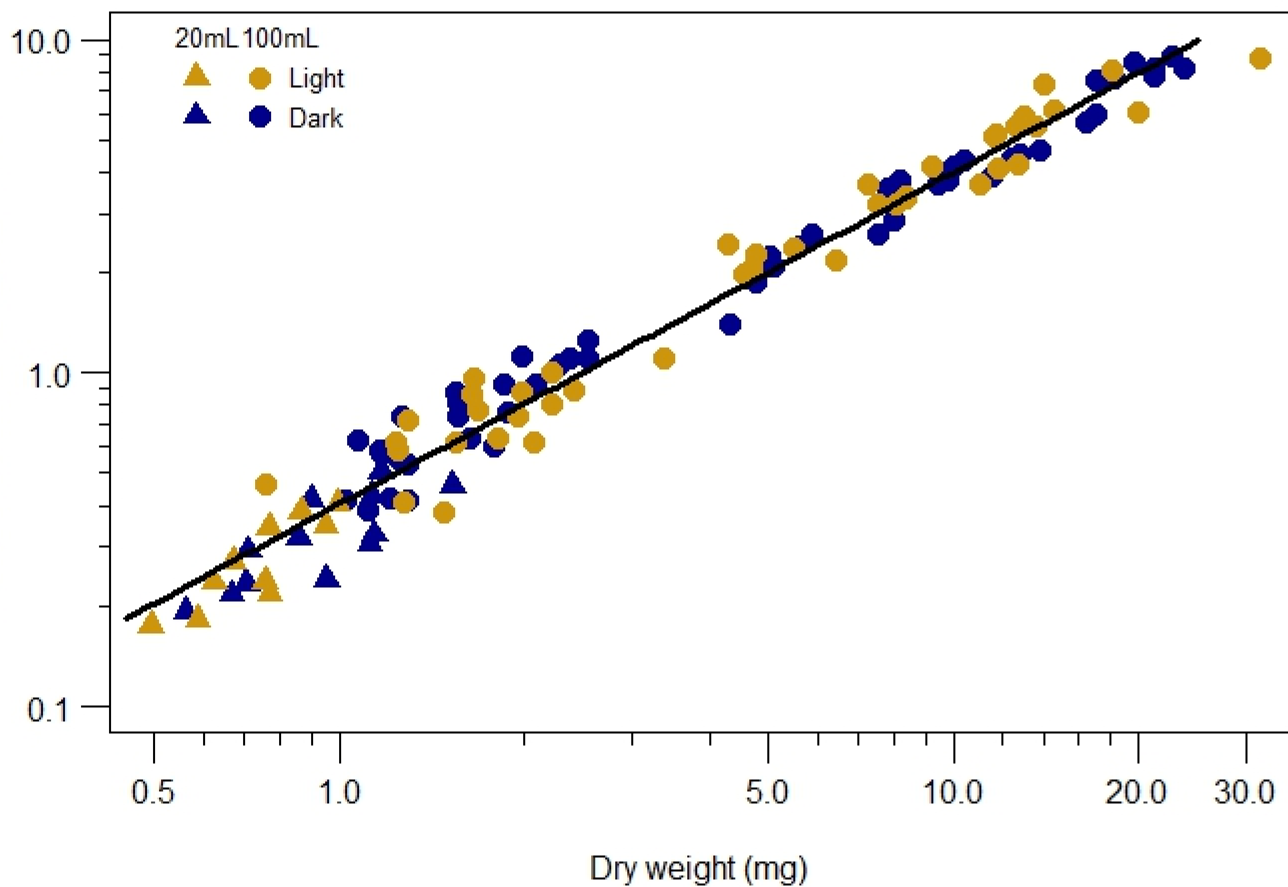

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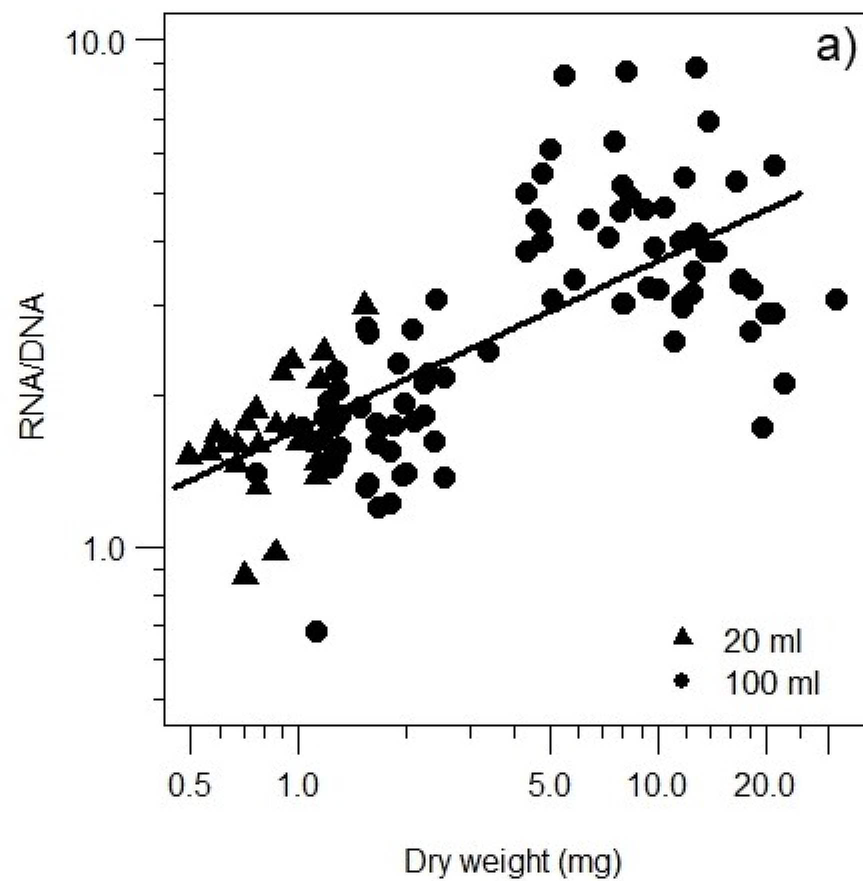

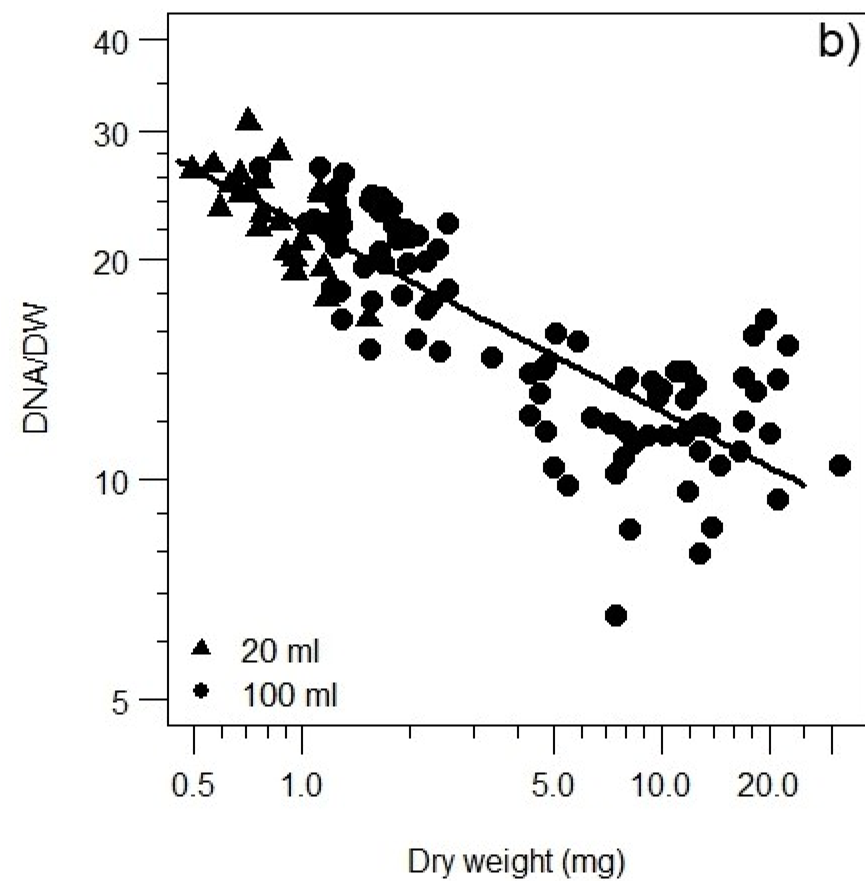

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