The effect of nutritional condition by two nucleic acid derived indices on the growth to post-flexion of Atlantic bluefin tuna and Atlantic bonito larvae

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ABSTRACT

Notochord flexion increases the swimming capacity of fish larvae, aids in the capture of mobile prey, and coincides with the timing of when the physiological capacities of larvae begin to develop significantly, allowing an early shift to piscivory. Therefore, reaching the flexion stage as soon as possible can be considered beneficial for the growth and survival of the larvae. Individual growth differences of larvae from the same cohort are very common before reaching flexion and the potential explanation is still unknown. In this study, we examined if the nutritional status of the larvae, measured by the RNA:DNA and DNA:DW ratios, explains the differences in the development of notochord flexion in laboratory-reared Atlantic bluefin tuna (ABFT; *Thunnus thynnus*) and Atlantic bonito (AB; *Sarda sarda*) larvae. Moreover, we described the ontogeny of the different stages and condition indices. The daily average condition of ABFT estimated by the RNA:DNA and DNA:DW ratios increased with larval age and developmental stage, whereas in AB it was not. In both species, bigger larvae at a specific age had higher nutritional condition than smaller larvae regardless of developmental stage. However, in ABFT the DNA:DW ratio indicated lower condition for the biggest larvae within developmental stages F0 (pre-flexion) and F3 (post-flexion). After removing the size effect, larger individuals of ABFT within flexion stages F1 and F2 had higher condition than any other larval stages, whereas in AB an indistinct trend within stages was found. Our results confirm that in ABFT the RNA:DNA ratio is correlated with faster development while the DNA:DW ratio is negatively correlated with the time to flexion. The trends found in the DNA:DW ratio points at cell growth mechanisms being a better indicator of larval condition that the RNA:DNA ratio. Our findings suggest that under culture, *ad libitum* conditions, larval nutritional conditions measured by nucleic acid ratios, might not be enough to explain developmental differences in fast growing species.

Key words: Atlantic bluefin tuna, Atlantic bonito, RNA-DNA ratio, DNA-DW ratio, fish larvae, development, flexion

1. Introduction

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). During their first weeks of life —a period characterized by high mortality rates (Bailey and Houde, 1989; Leggett and Deblois, 1994)— and after they have consumed all the lipids of the yolk, fish larvae have low energy reserves, and their tissues and organs are under progressive and intense differentiation and development (Fuiman, 1983; Kendall et al., 1984; Osse et al., 1995; Pittman et al., 2013).

Suboptimal feeding may lead to the death of larvae directly, by starvation, or indirectly, through prolonged stage duration and higher vulnerability to predation (Fiksen and Jørgensen, 2011; Folkvord et al., 2015). Differences in nutritional condition can also generate growth variability, and larvae of the same age may show individual differences, consequently influencing their success during this critical early life stage (Takebe et al., 2012; Tanaka et al., 2010).

One important event in the early life of fish is the flexion of the notochord, which accompanies the hypocordal development of the homocercal caudal fin during the larval stage (Kendall et al., 1984). This physiologically stressful moment is associated with changes in body shape, resulting in improvements in swimming capacity and feeding techniques (Kendall et al., 1984; Osse et al., 1995). It is also considered an inflexion point in the early stages of the life cycle of a fish, where growth and survival rates increase due

68 to enhanced access to a wider spectrum of prey and increased predation avoidance (Kaji,
69 2003; McFarlane et al., 2000; Somarakis and Nikolioudakis, 2010).

70 Atlantic bluefin tuna (ABFT; *Thunnus thynnus*), and Atlantic bonito (AB; *Sarda sarda*),
71 are the only scombrid species in the Mediterranean Sea that have been successfully reared
72 in captivity (De la Gándara et al., 2012; Ortega, 2015). Experimental studies have shown
73 that these two species become piscivorous already during the larval stage, once the flexion
74 of the notochord has occurred (Blanco et al., 2017; Reglero et al., 2014). This change in
75 diet increases survival and is required for sustaining growth and the high metabolic
76 requirements of these species (Reglero et al., 2014). The flexion coincides with the start
77 of the development of an adult-type digestive system, with blind sac, gastric glands,
78 pyloric caeca and digestive enzymes that allow for the digestion of fish prey (Kaji, 2003;
79 Miyashita et al., 1998; Yúfera et al., 2014). On the other hand, the completion of the
80 flexion is externally accompanied by the development of the fin rays and the development
81 of the caudal muscle fiber, which will allow for improved swimming (Kendall et al., 1984;
82 Osse et al., 1995; Roy et al., 2014). In general, bluefin tunas are also characterized by
83 having a very low tolerance to starvation, with up to 50% of the larvae dying one day
84 after starved conditions and showing an immediate growth retardation. Therefore, those
85 with low feeding incidence (or low nutritional condition) will rapidly die (e.g. Pacific
86 bluefin tuna, Tanaka et al., 2008). Growth differences in individual larvae from the same
87 cohort have been studied in Pacific bluefin tuna before reaching the flexion stage using
88 otolith back-calculation analyses in laboratory-reared and field-captured larvae (Takebe
89 et al., 2012; Tanaka et al., 2006). However, the effect of the nutritional condition in the
90 larval growth variability of tuna has only been studied in two laboratory studies using
91 stable isotope analyses in Pacific bluefin tuna larvae offered different preys (Tanaka et
92 al., 2010, 2014).

93 Inter-individual condition differences during the earliest life stages, have been largely
94 overlooked before now. The collection of post-flexion larvae in the field is a difficult task,
95 mainly due to their ability to evade sampling nets once their swimming capacity has
96 improved (Satoh et al., 2008). Additionally, assessing individual nutritional condition
97 from field samples is difficult, as extrinsic factors experienced by larval fish are often
98 changing, and different larvae of the same cohort might experience different
99 environmental conditions in a single day (Peck et al., 2012). Food availability and water
100 temperature are the main factors affecting the nutritional condition of fish larvae (e.g.
101 Buckley et al., 1984; Clemmesen, 1994; Foley et al., 2016; Folkvord et al., 1996).
102 Laboratory studies allow us to obtain larval sizes rarely captured in the field and to
103 identify individual responses in the nutritional condition to different feeding and thermal
104 controlled conditions.

105 Larval nutrition has been suggested as a possible cause of the high mortality in laboratory-
106 reared ABFT and AB (Reglero et al., 2014) but this has never been tested. To date, lipid
107 content and histology of the organs have been used as a proxy of nutritional condition in
108 ABFT (Ortega and Mourente, 2010; Yúfera et al., 2014). For Pacific bluefin tuna, the
109 ontogenetic changes in nutritional condition have been analyzed in the laboratory and in
110 the field (Tanaka et al., 2007, 2008), whereas data for ABFT is only available for field-
111 captured larvae (García et al., 2006). Nutritional condition is associated with the food
112 supply and feeding success of the fish and therefore variability in the trophic environment
113 is reflected in nutritional condition.

114 In this study, we examined if the nutritional status of laboratory reared ABFT and AB
115 larvae could explain individual differences in the timing of notochord flexion. We use
116 two different nucleic acid derived indices to determine larval fish nutritional condition:
117 the RNA:DNA and the DNA:dry weight (DNA:DW) ratios. The RNA:DNA ratio is an

118 index of cell metabolic intensity and it is used as an approach for recent growth and recent
119 nutritional condition of fish larvae (Clemmesen, 1994; Folkvord et al., 1996). The amount
120 of DNA is stable under changing environmental situations, reflecting the number of cells
121 of an individual, whereas the amount of RNA is directly proportional to the protein
122 synthesis capacity in the cell. The RNA is highly dependent on food quantity and varies
123 with age, life stage, organism size, disease state, changing environmental conditions and
124 diel differences (Buckley et al., 1999; Rooker and Holt, 1996). During food deprivation,
125 the nutritional condition, and therefore the RNA:DNA ratio, decreases, reflecting the
126 cessation of protein synthesis and somatic growth (e.g. Clemmesen, 1994). Therefore,
127 well-fed larvae are metabolically more active, grow faster and have relatively higher
128 RNA:DNA ratios compared to poor-fed larvae with less active metabolism (Clemmesen,
129 1987, 1994). The RNA:DNA ratio is sensitive to changes in specific growth rates, both
130 in terms of length and weight, and provides information on the feeding environment of
131 the larvae within a time frame of days prior to sampling. This can be interpreted as recent
132 growth capacity and can be useful to examine the survival processes (Bergeron, 1997;
133 Rooker and Holt, 1996). On the other hand, the DNA:DW ratio indicates the cell
134 condition, since cell weight decreases while the amount of DNA remains constant during
135 a reduction in the nutritional condition of the larvae (Bergeron, 1997). Opposite, the
136 DNA:DW ratio increases when nutritional condition decreases, since more cells are
137 present for the same weight of tissue of starved larvae (Bergeron, 1997; Chícharo and
138 Chícharo, 2008). Some authors consider being the DNA:DW ratio a more stable and
139 sensitive ratio during the early stages than the RNA:DNA ratio due to the high variability
140 of RNA content during the larval stage (Bergeron, 1997).

141 In this study we sought to determine the effect of the nutritional condition on larval
142 growth and developmental variability to post-flexion stage and to describe the ontogeny

143 of the different stages and condition indices in ABFT and AB larvae. Two nucleic acid
144 derived indices, RNA:DNA and DNA:DW ratios, were used as condition measures to test
145 the hypotheses that 1) at a specific day, larvae with higher nutritional condition were
146 bigger in size at any developmental stage than the smaller larvae with lower nutritional
147 condition and 2) at a specific size, more developed larvae had higher nutritional condition
148 than the less developed ones.

149 2. Material and methods

150 2.1. Atlantic bluefin tuna experiment

151 Fertilized eggs of ABFT were collected from naturally-spawning captive adults in the
152 farming facilities at El Gorguel, Cartagena (SE Spain), owned by Caladeros del
153 Mediterráneo S.L. In the laboratory, floating and sinking eggs (at natural seawater salinity
154 of 37) were separated in a 5 L bucket. Floating ABFT eggs were incubated in 400 L tanks
155 at 28 °C under a continuous light regime. A few hours after incubation, 60000 ABFT eggs
156 were transferred to four 1500 L cylindrical tanks, with 15000 eggs per tank and the sea
157 water temperature set at 28 °C. Assuming a hatching rate of 85-90% (personal
158 observation), initial larval density ranged between 8.5-9 larvae L^{-1}. Water temperature in
159 the incubators and in the rearing tanks was controlled using heaters—isolated to avoid
160 larval mortality—inside the tanks. The water temperature was measured continuously by
161 a HOBO data logger (www.onsetcomp.com). Rearing was conducted with a photoperiod
162 of 14L:10D, similar to natural conditions in the area.

163 Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day from 0
164 to 3 days post hatch (dph). Afterwards, a paste of concentrated *Chlorella* (Super fresh
165 Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) was added three times per day in

each tank. The larvae were fed following the technique described in De la Gándara et al. (2012). Enriched rotifers, *Brachionus plicatilis*, were added from 3 dph at a concentration of 5 rot mL^{-1} to guarantee *ad libitum* conditions. From 14 dph onwards, gilthead sea bream (*Sparus aurata*) yolk-sac larvae of 0–2 dph (3.4 ± 0.04 mm) were added, providing up to 300 preys per individual twice daily. Pseudo–green water technique was used during the entire rearing period to avoid the depletion of the nutritional condition of the rotifers and the resulting effect on larval development (Yamamoto et al., 2009).

Several small samplings were carried out every day before 8 dph in ABFT to follow the development of the larvae and to accurately identify the first day any larvae started to flexion. Experimental samplings started as soon as we found flexion stage larvae and finished when at least 50% of the larvae were found to be in the post-flexion stage to minimize the possibility that cannibalism might result in differential feeding. The experiment lasted for 20 days, from 0 to 20 dph. 40 larvae per tank were randomly sampled at 8, 10, 11, 12 and 13 dph. At 13 dph, all the remaining ABFT larvae in the tanks were counted to estimate survival and a total of 1845 larvae were transferred to three new 1500 L cylindrical tanks, with up to 615 larvae randomly distributed in each tank. Hereafter, the larvae were cultivated until they were 20 dph and reached the early juvenile stage, at which point 40 larvae per tank were sampled and all the remaining larvae were counted for survival estimates. Larval sampling was carried out every day at the same time, early in the morning and in darkness using a long siphon whose diameter was increased according to larval sizes. By using the siphon, we ensured the sampling of the weaker (probably in the upper part, easy to catch) and stronger (probably in the bottom, hard to catch) larvae.

2.2. Atlantic bonito experiment

190 Fertilized eggs of AB were obtained from stripped spawning adult individuals collected
191 in an almadraba trap in La Azohía (Murcia, SE Spain). In the laboratory, floating and
192 sinking eggs (at natural seawater salinity of 37) were separated in a 5 L bucket. Floating
193 AB eggs were incubated in 400 L tanks at 26 °C under a continuous light regime. Just
194 after hatching, at 0 dph, 11250 AB larvae were transferred to three 1500 L cylindrical
195 tanks, with 3750 larvae in each tank, 2.5 larvae L^{-1}, and the water temperature set at 26
196 °C. Water temperature in the incubators and in the rearing tanks was controlled using
197 heaters—isolated to avoid larval mortality—inside the tanks. The water temperature in
198 each tank was measured continuously by a HOBO data logger (www.onsetcomp.com).
199 Rearing was conducted with a photoperiod of 14L:10D, similar to natural conditions in
200 the area.

201 Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day, from 0
202 to 2 dph. Afterwards, a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12,
203 Chlorella Industry Co., Ltd., Japan) was added three times per day in each tank. The
204 larvae were fed following the technique described in De la Gándara et al. (2012). Enriched
205 rotifers, *Brachionus plicatilis*, were added from 2 dph at a concentration of 5 rot mL^{-1} to
206 guarantee *ad libitum* conditions. Pseudo-green water technique was used during the entire
207 rearing period to avoid depletion of the nutritional condition of the rotifers and the
208 resulting effect on larval development (Yamamoto et al., 2009).

209 Several small samplings were carried out every day before 5 dph to follow the
210 development of the larvae. Experimental samplings started as soon as we found flexion
211 stage larvae and finished when at least 50% of the larvae were found to be in the post-
212 flexion stage, to avoid the possible cannibalism of those that first reached the post-flexion
213 stage. The experiment lasted for 9 days, from 0 to 9 dph. 20 larvae were randomly
214 sampled in each tank at 5, 7 and 8 dph while 30 larvae per tank were sampled at 6 dph

(Table 1). The last day, 9 dph, all the remaining larvae in the tanks were counted and sampled. Larval sampling was carried out every day at the same time, early in the morning and in darkness.

2.3. Laboratory analyses

Immediately after sampling, the larvae were anesthetized using clove oil (Guinama© Spain), individually photographed using an image analysis system connected to a microscope (Leica Microsystem, Inc, Bannockburn, IL) and individually frozen in vials at -80 °C. ABFT larvae were submerged in RNAlater® before preserving at -80 °C. Later, in the laboratory, the larvae conserved in RNAlater® (Sigma-Aldrich R0901) were rinsed with milliQ water and lyophilized to estimate individual dry weight (DW) (to the nearest 0.01 mg) and larval nutritional condition. The standard length (SL) of the sampled fish was measured to the nearest 0.1 mm from the anterior margin of the snout to the posterior margin of the hypural plate of the notochord. Four different developmental phases based on morphological characteristics of the notochord and caudal fin were determined following a modified version of the criteria of De la Gándara et al. (2013), a modified version for *Thunnus thynnus* of Kendall et al. (1984) and Kaji et al. (1996) (Table 2): 1) larvae in pre-flexion (F0), 2) larvae with development of the first caudal fin rays (F1), 3) larvae in flexion (F2) and 4) larvae in post-flexion (F3).

2.4. Nutritional condition: nucleic acid analyses

RNA:DW and DNA:DW ratios were determined using a modification of the method described by ICES (2004). RNA:DW and DNA:DW were individually measured using the whole larval body and all the reagents were prepared using Tris-EDTA buffer (0.05 M TRIS, 0.1 M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl). First, lyophilized larvae were rehydrated by transferring to a mixture of Tris-EDTA buffer and sodium

239 dodecyl sulfate 0.7% (SDS) for 15 minutes at 4 °C. Once rehydrated in the vial, the larvae
240 were completely disintegrated by applying two 10-second ultrasound pulses (Bandelin
241 Sonoplus). An increase in the temperature of the homogenates was avoided by keeping
242 all the vials on ice. The homogenate was centrifuged at 3800 x *g* during 8 minutes at 4
243 °C. Two supernatant aliquots were taken, one for the measurements of the total nucleic
244 acids (RNA + DNA) and another one for the measurement of the DNA content (µg fish^{-1}).
245 The DNA measurement was carried out by incubating the samples with RNase A (type
246 I-AS, Sigma-Aldrich) at 37 °C during 30 minutes. The difference between the total
247 nucleic acids fluorescence and the DNA fluorescence was corrected to determine the
248 RNA fluorescence as suggested by Caldarone et al. (2006) assuming for DNA, a ratio of
249 2.4 RNA content (µg fish^{-1}).

250 Nucleic acids fluorescence was determined fluorometrically with a Perking-Elmer LS-5
251 (excitation: 327 nm and emission: 614 nm) by adding 200 µL Ethidium Bromide buffer
252 solution (0.1 mg mL^{-1}). DNA and RNA content were estimated by means of calibrated
253 standards curves of calf thymus DNA (Sigma-Aldrich) and baker yeasts RNA (Sigma-
254 Aldric), respectively. All biochemical analyses of larvae reported in this study were
255 completed within 4–5 months after sampling.

256 2.5. Statistical analyses

257 All the statistical analyses were carried out using the R statistical software package
258 (version 3.4.3, Development Core Team, 2017). Final survivals and daily mortalities were
259 estimated from the numbers of initial eggs and the number of larvae counted out at the
260 end of the experiment, subtracting the number of larvae sampled on each sampling day.
261 All size data (SL, DW) were analyzed for heterogeneity of variance (Levene's test) and
262 checked for normality with a Kolmogorov–Smirnov test.

263 Differences in larval sizes (SL, DW) among replicates within each species were tested
264 using one-way ANOVA, and Bonferroni correction was applied to avoid type I error. A
265 two-way ANOVA test was performed in each one of the 5 studied variables (RNA:DNA
266 ratio, RNA:DW ratio (ug mg^{-1}), DNA:DW ratio (ug mg^{-1}), standard length (mm) and dry
267 weight (mg)) with stage and dph as factors. And when significant, Tukey HSD tests were
268 used for post-hoc comparisons. All test results were considered significant at a level of
269 0.05.

270 Cumulative size distributions (CSDs) in standard length and dry weight were estimates
271 as described in Folkvord et al. (2009). Assuming static ranking of fish sizes within a
272 cohort is unlikely to change much in the short term, cumulative size distributions were
273 used for visualizing growth variabilities within cohorts over time in a single graph and
274 can also reveal size-dependent mortality among sampling days (Folkvord et al., 2009).
275 Stage developmental cumulative distribution was also estimated in order to obtain a
276 cumulative approach of the duration of the different developmental stages in each species.

277 The residuals from the nucleic acid ratios RNA:DNA relationship (RNA-DNA residuals)
278 and DNA:DW relationship (DNA-DW residuals), were compared to the dry weight *vs*.
279 dph (size-at-age) relationship residuals (DW-DPH residuals). A significant positive
280 (negative) correlation indicates faster growing larvae have higher (lower) nutritional
281 condition index than slower growing larvae. In order to determine if the nutritional
282 condition can explain the differences of having different developmental stages at a
283 specific size, the residuals of both nutritional conditions were analyzed against the stage
284 *vs*. dry weight relationship residuals (STAGE-DW residuals). Residuals were analyzed
285 using linear regression and ANCOVA analyses were carried out for stage effect.

286 ## 3. Results

287 There were no significant differences in the daily larval sizes among tank replicates in
288 both species (ANOVA, *p-adj.*>0.05); therefore, replicates were combined for further
289 analyses. Survival rates of ABFT at 13 dph and AB at 9 dph were 4.1 ± 0.2% and 1.1 ±
290 0.2% respectively. Further, 27.7 ± 2.5% of the ABFT larvae survived from 13 to 20 dph.
291 On average, every day 23.6 ± 6.4% of ABFT larvae died up to 13 dph, and 16.7 ± 1.0%
292 up to 20 dph. In AB, 39.2 ± 1.6% died daily until 9 dph.

293 Near-parallel CSDs among subsequent sampling days showed similar growth rates of
294 different size-ranked ABFT and AB larvae both in length (Fig. 1a, c) and dry weight (Fig.
295 1b, d). However, AB larvae showed lower growth rates from 8 to 9 dph than at other age
296 intervals, as indicated by the almost overlapping cumulative curves (Fig. 1c, d).

297 There was no significant overlap in the larval length and weight among developmental
298 stages both in ABFT and AB (Fig. 2, Table 3, Table 4, ANOVA, Tukey HSD, $p<0.05$).
299 ABFT larvae showed the first signs of flexion (stage F2) from 6.1 mm in length and 0.24
300 mg in weight and completed flexion (stage F3) from 6.5 mm in length and 0.43 mg in
301 weight (Table 3). In AB first flexion (stage F2) larvae were found from 6.3 mm in length
302 and 0.24 mg in weight and completed flexion from 8.2 mm in length and 0.80 mg in
303 weight (stage F3) (Table 3).

304 There was an overlap in the age at which different developmental stages were observed
305 (Fig. 3). In ABFT, F0 stage larvae lasted to 11 dph, while in AB F0 larvae lasted only to
306 6 dph (Fig. 3). The start of F1 stage was not determined but the development of the rays
307 in the last caudal fin was observed until 13 dph in ABFT and 8 dph in AB. Initial flexion
308 (F2) in ABFT was observed from 8 dph and post-flexion from 9 dph (Fig. 3a). In AB, F0
309 stage finished around 6–7 dph and F1 at 8 dph. First flexion AB larvae started from 5 dph
310 and post-flexion AB larvae from 7 dph (Fig. 3b). In ABFT, at 10 and 11 dph, larvae of

311 all the stages co-existed, while in AB, at day 6 dph the larvae of first three the stages co-
312 existed.

313 We found an increasing trend in the daily average RNA:DNA ratio with age in ABFT,
314 whereas a steady tendency was observed in AB independent of age (dph) (Fig. 4). In
315 ABFT daily average RNA:DNA ratio was related to the DNA:DW ratio, since RNA:DW
316 ratio remained steady during the experimental period (Fig. 4a). The daily average
317 DNA:DW ratio decreased with age and developmental stage in ABFT (Fig. 4b), resulting
318 in an increase of the RNA:DNA ratio with age and developmental stage (Fig. 4c, Table
319 4, ANOVA, Tukey HSD, $p<0.05$). In AB, RNA:DNA, RNA:DW and DNA:DW ratios
320 varies with larval age caused by the high variability found in the first sampling days (Fig.
321 4d, f, Table 4, ANOVA, Tukey HSD, $p<0.05$) although they did not vary with
322 developmental stage (Fig. 4d, f , Table 4, ANOVA, $p>0.05$).

323 There was a positive correlation between larval RNA-DNA residuals and DW-DPH
324 residuals (and a corresponding negative correlation between DNA-DW and DW-DPH
325 residual) in ABFT and AB (Supplementary Fig.1a, b, c, ANCOVA, $p<0.05$). Bigger sized
326 larvae at a specific age, had higher nutritional condition than small sized larvae regardless
327 the developmental stage (ANCOVA, $p>0.05$). However, in ABFT, the DNA:DW ratio
328 showed a significative positive correlation (decrease nutritional condition) within stages
329 F0 and F3 (Fig. 5, ANCOVA, $p<0.05$). Bigger sized larvae at a specific age within stages
330 F0 and F3 in ABFT had the lower nutritional condition.

331 The size effect of the larvae was removed by analyzing the condition ratios residuals
332 against larval STAGE-DW residuals (stage-at-size). In ABFT relatively larger larvae
333 within stages F1 and F2 showed a significative higher nutritional condition with regard
334 the other stages (Supplementary Fig. 1d, e, ANCOVA, $p<0.05$). However, in AB no

335 relationship was found analyzing DNA-DW residuals (Supplementary Fig.1 f,
336 ANCOVA, $p>0.05$) whereas RNA-DNA residuals showed that relatively larger larvae
337 within F1 stage had higher nutritional condition than the rest of the larvae of any other
338 stage (Fig. 6, ANCOVA, $p<0.05$).

339 ## 4. Discussion

340 Understanding what causes developmental variability in fish larvae is fundamental since
341 high mortality during the first days will determine the number of individuals that would
342 reach the juvenile stage being particularly important in those species where inter-
343 individual variability can lead to cannibalism. The nucleic acid ratios have been widely
344 used to determine the nutritional condition of fish larvae. In this study, we explore if the
345 nutritional condition of the larvae explains differences in the age, size and stage at which
346 development of the notochord occurs during the first days of life in ABFT and AB larvae.
347 We found that the nutritional condition is related with larval growth in both species. At
348 specific size, more developed larvae are related with higher nutritional condition in
349 ABFT, but not in AB.

350 In our study we have estimated survival rates of 1% and 4%, for AB and ABFT
351 respectively, for the period between hatching and post-flexion, and survival rates of 28 %
352 from post-flexion to juvenile in ABFT. Survival rates for laboratory-reared Pacific bluefin
353 tuna and yellowfin have been reported between the time from hatching to juvenile,
354 varying between 0.07% and 3% (Margulies et al., 2007, 2016; Sawada et al., 2005;
355 Tanaka et al., 2018), and from post-flexion to the end of the piscivory phase between 30%
356 and 60% (Blanco et al., 2017; Reglero et al., 2014; Seoka et al., 2008; Tanaka et al.,
357 2014). In AB, survival from hatching to juvenile varies between 2.9% and 10% (Blanco
358 et al., 2017; De la Gándara et al., 2012; Reglero et al., 2014). These survival data, in

359 accordance with other laboratory data reported, suggest once the larvae reach flexion, the
360 mortality rates decrease significantly. The high mortalities observed during the first days
361 of life in laboratory reared Pacific bluefin tuna or yellowfin have been related to culture
362 techniques that are still in development (Honryo et al., 2016; Nakagawa et al., 2011;
363 Tanaka et al., 2008, 2009), malnutrition (De la Gándara et al., 2012; Margulies et al.,
364 2016; Takebe et al., 2012), adhesion to the surface and to the sinking syndrome (Sawada
365 et al., 2005; Takashi et al., 2006; Tanaka et al., 2009). Besides, rotifers are not the natural
366 preys of tuna larvae from the field which can have an effect in larval survival. Copepods
367 are one of their natural preys and larval survival is known to be improved in comparison
368 with rotifers (Llopiz and Hobday, 2015; Ortega, 2015).

369 The completion of the head for feeding and respiratory functions, the tail for cruising and
370 escape reactions and the full development of the intestine appears to be given priority
371 during the first days of life rather than growth in total body length (Osse et al., 1995).
372 However, as seen from the successive CSDs in larval size, we found similar daily specific
373 growth rates in standard length and dry weight in ABFT and AB with no size-dependent
374 mortality event. The changes in notochord flexion are accompanied by the development
375 of fin rays, changes in body shape, locomotive ability, and feeding techniques (Kendall
376 et al., 1984). Flexion can be used as a proxy for the development of other non-visible
377 changes, such as the appearance of the first gastric glands, the complete development of
378 the stomach with the first pyloric caeca and the development of pharyngeal teeth (Yúfera
379 et al., 2014). Size instead of age is a good proxy for morphological development, since,
380 as we found, there is an overlap in the age at which different developmental stages were
381 observed, while there is no strong overlap in the larval length and weight among
382 developmental stages. The vast majority of the studies regarding tuna larval development
383 are mainly focused on the development of digestive physiology (e.g. Buentello et al.,

384 2011; Miyashita et al., 1998), organogenesis (e.g. Fujimoto et al., 2008; Yúfera et al.,
385 2014) and the development of morphological structures (e.g. Miyashita et al., 2001). Our
386 results from the laboratory indicate first flexion signs from 6.1 mm and first post-flexion
387 signs from 6.5 mm, similar to those reported at 5–5.7 mm and 7–7.4 mm in Pacific bluefin
388 tuna at temperatures of 24.5–27.7 °C and 25 ºC, respectively (Kaji et al., 1996; Miyashita
389 et al., 2001).

390 By the boxplot analyses we found that daily average RNA:DNA ratio during the first days
391 (8-13 dph) varies with the age and development in ABFT larvae. The increasing trend
392 values is correlated with the decreasing DNA:DW ratio and constant RNA:DW ratio with
393 age and development. Same trends have been obtained during the ontogenetic
394 development of other non scombrid species (Bergeron, 1997; Malzahn et al., 2003). The
395 decreasing trend in the DNA:DW ratio suggests a switch in the growth mechanisms of
396 the larval cells from a higher proportion of hyperplasia to a higher proportion of
397 hypertrophy (Buckley et al., 1999; Malzahn et al., 2003). Hyperplastic growth occurring
398 by proliferation of new cells is characterized by mitotic activity, whereas hypertrophy is
399 the enlargement of the existing cells (Weatherley et al., 1988). A decrease in the
400 DNA:DW ratio is achieved by a higher increment in the body weight of the larvae (DW)
401 in relation with the amount of DNA present (genetic material), suggesting a switch to cell
402 enlargement (hypertrophy). Therefore, our results suggest that once the larvae have
403 completed the post-flexion stage and started piscivory, growth by cell enlargement
404 dominates, a trend that has been observed in the juvenile stage of Pacific bluefin tuna
405 (Tanaka et al., 2007). Evident hypertrophy from histochemical analyses has been
406 documented from 29 dph in Pacific bluefin tuna juveniles, indicating hyperplasia
407 persisted over a long period of time (Roy et al., 2012, 2014). In AB larvae, daily average
408 DNA:DW ratios remained unchanged with development but not with the age (probably

409 caused by the high variability in some days) which may be explained by an earlier
410 combination of hyperplasia and hypertrophy in AB compared to ABFT larvae. The
411 dynamic of hyperplasia and hypertrophy has been seen to determine the ultimate somatic
412 size of the fish. In small adult size species hyperplasia ceased early and most of the growth
413 is attributed to hypertrophy, whereas in those attaining large adult sizes hyperplasia
414 continued for a long time in the development (Weatherley et al., 1988). AB adult sizes
415 are much smaller than those of ABFT, which may explain the possible difference in cell
416 growth mechanisms.

417 The decreased tendency of average DNA:DW ratio with age (and size) might be due to
418 artifacts during the homogenization and sub-sampling protocol. Larger larvae may be
419 more difficult to homogenize by sonication, and thus, they might not be completely
420 digested by the time of the sub-sampling of the homogenate. In this case, the supernatant
421 sub-sample in larger larval homogenates might contain less nucleic acid than the
422 supernatant from smaller, more easily digested larvae and the sub-sample might not
423 represent the original larval dry weight. Moreover, it is possible that some tissue types
424 are digested more easily than others and this would also get a bias sample of some tissue
425 types in greater proportion than others, with potential differences in nucleic acid ratio in
426 those different tissues (Olivar et al., 2009). The higher average RNA:DNA ratio values
427 in ABFT than AB may be explained by the high proportion of the head tissue into the
428 biochemical analyses, resulting in a decrease in the total RNA:DNA ratio. The
429 RNA:DNA ratio in the head of several species is lower than in the muscles due to higher
430 DNA:DW ratio derived from the presence of a larger number of small cells in the head
431 (Olivar et al., 2009). Olivar et al. (2009) also suggested that the lower RNA:DNA ratio
432 in the head than in muscle could indicate lower growth rates than muscle tissues related
433 to lower protein synthesis. AB larvae are characterized for having a very big head,

representing more than a third of the body size, until reaching the juvenile stage, whereas ABFT's head is also bigger than in other larval fish species, but proportionally smaller than in AB (Rodríguez et al., 2017).

A size effect (positive correlation) was found by the residual analysis of RNA:DNA ratio and larval size (or growth) at specific age in both species, where bigger larvae had a higher nutritional condition, and also suggesting good larval condition in ABFT and AB larvae during the five days or more before sampling. However, the cell condition ratio, DNA:DW residuals in ABFT showed a decrease in the cell condition within the developmental stages F0 (pre-flexion) and F3 (post-flexion), not in AB. The biochemical measures of cell size (DNA:DW ratio), rather than the protein synthesis capacity (RNA:DNA ratio), was a better indicator of the condition of the different flexion stages in ABFT larvae (Bergeron, 1997). In ABFT larvae, where more larvae were sampled, the RNA:DNA ratio measurements were highly variable, stemming from the high variability obtained in the RNA. The RNA:DNA ratio might not be as strongly related to larval feeding condition as it is the DNA:DW ratio which is considered more stable. It may also be more sensitive to the nutritional status of the larvae because cell weight is decreasing while the amount of DNA is kept constant if feeding decreases (Bergeron, 1997). Besides, the DNA:DW ratio may be more related to the larval stage of fishes while the RNA:DNA ratio seems to be more related to feeding condition during the late larval of juvenile stage as was pointed out in Bergeron (1997).

Since there is a size effect on the nutritional condition of the larvae, the nucleic acid ratios against the developmental stage at a specific size were used to determine if the nutritional condition is responsible for the different developmental individuals at the same size. In ABFT, in general, we found that at a given size more developed larvae are related with higher nutritional condition and within each developmental stage, nutritional condition of

459 the relatively larger larvae in flexion stages (F1 and F2) was higher than those in pre-
460 flexion (F0) and post-flexion (F3). Fast growth during the first days of life (F0 stage)
461 along with a possible suboptimal feeding shortly after yolk absorption, may be
462 responsible of the decrease in the nutritional condition of the larvae. We saw the first
463 signs of post-flexion at 10 dph while the larvae offered as prey was first offered to the
464 tanks at 13 dph. The timing to switch to piscivory in ABFT is known to determine further
465 larval growth and survival and a delay of 4 or 8 days can increase mortalities (Reglero et
466 al., 2014). The delay in prey switch in our experiments may have affected the nutritional
467 state of those larvae already in post-flexion (F3) at 10 dph (Takebe et al., 2012). This
468 might be apparent in the nucleic acids (fast response) but not yet in the larval weight. The
469 suboptimal feeding of larvae in post-flexion might be hiding an increase in RNA:DW
470 ratio once the notochord flexion is completely finished. The increased energy demands
471 due to the active swimming of the post-flexion larvae might exceed the reduced energy
472 gain during suboptimal feeding decreasing larval condition (Billerbeck et al., 2001; Faria
473 et al., 2011; Illing et al., 2018; Lankford et al., 2001; Moyano et al., 2018). Silva et al.
474 (2015) in plaice larvae (*Pleuronectes platessa*) concluded that larvae with lower
475 DNA:DW ratio had better swimming abilities, however they did not find a relationship
476 with the RNA:DNA ratio. In AB at a given size, relatively larger individuals had higher
477 nutritional condition only within stage F1. The decrease in growth seen from 8 to 9 dph,
478 might show how the delay in prey switch in our experiments might have affected the
479 nutritional state of those larvae at more advanced stages.

480 Our results clearly indicate that when parameters such as diet and temperature do not
481 vary, cell growth mechanisms are species-specific. Under culture, *ad libitum* conditions,
482 as seen in our results, feeding conditions obtained from nucleic acid derived indices might
483 not be enough to explain developmental differences in ABFT and AB larvae. The

484 explanation of growth differences and developmental rates in those fast-growing fish
485 larval species might be difficult to identify since short time intervals separate different
486 stages and differences in growth strategies between individuals (Juan-Jordá et al., 2013).
487 The nutritional condition measured by the nucleic acid derived indices RNA:DNA and
488 DNA:DW ratios are only two measures of biomolecular condition and other condition
489 analyses such as lipid levels or histological indices along with other aspects such as
490 energy consumption (e.g. swimming, specific dynamic action) should be investigated in
491 order to better understand larval species-specific strategies. As seen in other pelagic larval
492 species, the physiological response to nutrition level can be translated to affect the
493 viability of the larvae after handling (cod, Øie et al., 2015), regulate swimming and
494 metabolic rates (herring, Illing et al., 2018) and has a potential long-term effect in growth
495 and condition when reaching the juvenile stage (herring, Folkvord et al., 2018).

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741 *dumerili*. Fish. Sci. 75(3), 697−705.

742 Yúfera, M., Ortiz-Delgado, J. B., Hoffman, T., Siguero, I., Urup, B., Sarasquete, C., 2014.
743 Organogenesis of digestive system, visual system and other structures in Atlantic

744 bluefin tuna (*Thunnus thynnus*) larvae reared with copepods in mesocosm system.
745 Aquaculture 426, 126−137.

746 Table 1. Summary of the differences between Atlantic bluefin tuna and Atlantic bonito
747 experiments. Daily total number of larvae sampled (n) in each tank replicate (R1, R2, R3
748 and R4) per larval day post hatch (dph). Average temperature in °C during the
749 corresponding days and the average daily standard length (SL, mm) and dry weight (DW,
750 mg) of the sampled larvae is shown in the table. In Atlantic bluefin tuna at 20 dph, the
751 standard length is shown as a range due to the high size variability. Variability around the
752 mean is displayed as standard deviation (± SD).

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Species	Dph	Sampling (n)				SL (mm)	DW (mg)
		R1	R2	R3	R4		
Atlantic bluefin tuna	8	40	40	40	40	5.1 ± 0.5	0.13 ± 0.05
27.7 ± 0.4 °C	10	40	40	40	40	6.2 ± 0.7	0.32 ± 0.15
	11	40	40	40	40	6.7 ± 0.6	0.42 ± 0.18
	12	40	40	40	40	7.0 ± 0.7	0.58 ± 0.26
	13	40	40	40	40	7.5 ± 0.6	0.77 ± 0.26
	20	40	40	40	0	11.3 - 24.6	4.46 - 50.1
Atlantic bonito	5	20	20	20	0	5.7 ± 0.3	0.16 ± 0.03
26.2 ± 0.9 °C	6	30	30	30	0	6.6 ± 0.5	0.28 ± 0.06
	7	20	20	20	0	7.3 ± 0.4	0.45 ± 0.09
	8	20	20	20	0	8.0 ± 0.3	0.68 ± 0.13
	9	all	all	all	0	8.2 ± 0.3	0.77 ± 0.13

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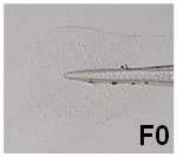
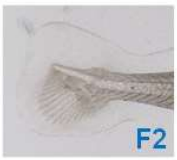
759 Table 2. Description of the four different developmental stages used in this article, based

760 on morphological characteristics of the notochord and caudal fin rays.

Stage	Nomenclature	Description	Example
Pre-flexion	F0	Straight notochord	
First caudal fin rays	F1	Straight notochord with some rays in the ventral side	
Flexion	F2	Bending upward of the notochord tip in a very clear angle with an increase in the amount of fin rays	
Post-flexion	F3	The final tip of the notochord disappears. Definition of the hypural plate and caudal fork. The posterior margin of the upper hypural plate is at 90 ° from the notochord axis	

761

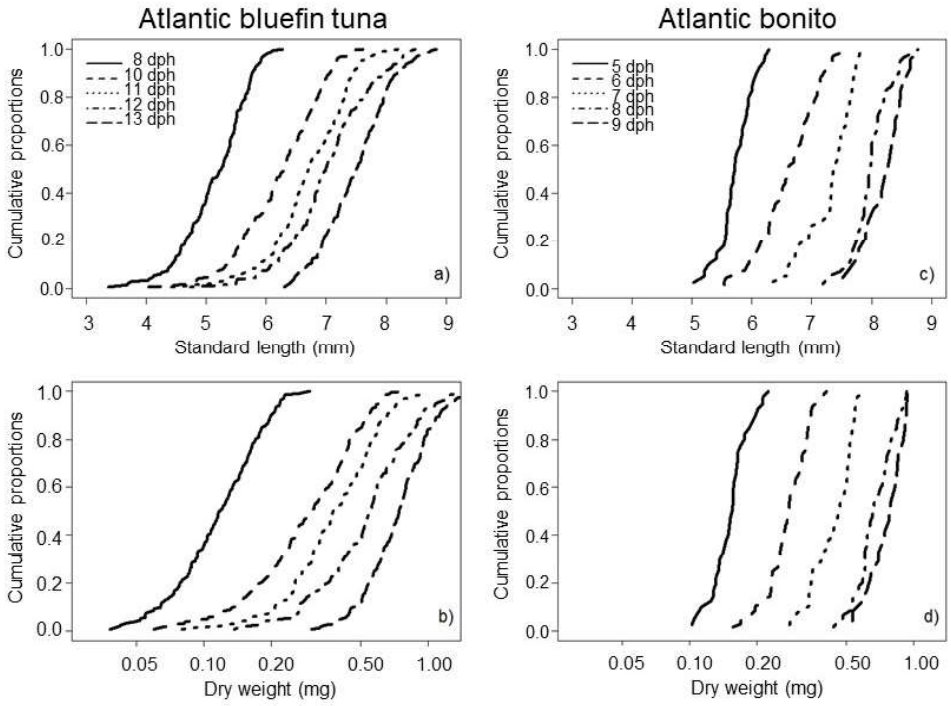
762 Table 3. Mean (± SD) standard length (SL, mm) and dry weight (DW, mg) measures of
763 each developmental stage (F0, F1, F2 and F3) found of Atlantic bluefin tuna and Atlantic
764 bonito larvae during our experiment. The exact moment the first larva in flexion (F2) and
765 in post-flexion (F3) was seen is documented, and the standard length and dry weight of
766 that larva is show as *first signs*.

Species	Measurements	F0	F1	F2		F3	
		average	average	average	first signs	average	first signs
Atlantic bluefin tuna	SL (mm)	4.8 ± 0.5	5.8 ± 0.4	6.7 ± 0.3	6.1	7.5 ± 0.5	6.5
	DW (mg)	0.10 ± 0.03	0.22 ± 0.08	0.43 ± 0.10	0.24	0.77 ± 0.24	0.43
Atlantic bonito	SL (mm)	5.4 ± 0.3	6.5 ± 0.6	7.8 ± 0.5	6.3	8.4 ± 0.2	8.2
	DW (mg)	0.1 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.24	0.90 ± 0.05	0.80

767 Table 4. Summary table of the results from the two-way analysis of variance (ANOVA)
768 among the studied variables. In Atlantic bluefin tuna, analyses were done from 8 to 13
769 dph and in Atlantic bonito from 5 to 9 dph.

	Atlantic bluefin tuna Pr (>F)	Atlantic bonito Pr (>F)
RNA/DNA		
dph	<0.001	<0.001
stage	<0.001	0.335
RNA/DW (μg mg^{-1})		
dph	0.323	<0.001
stage	0.023	0.378
DNA/DW (μg mg^{-1})		
dph	<0.001	<0.001
stage	<0.001	0.099
Standard length (mm)		
dph	<0.001	<0.001
stage	<0.001	<0.001
Dry weight (mg)		
dph	<0.001	<0.001
stage	<0.001	<0.001

770 Figure 1. Cumulative size distribution proportions of standard length (SL, mm) and dry
771 weight (DW, mg). a) Atlantic bluefin tuna SL, b) Atlantic bluefin tuna DW, c) Atlantic
772 bonito SL, and d) Atlantic bonito DW. Different experimental samplings days are shown
773 with different cumulative line type. Note: The x-axis is log transformed for DW in panels
774 b) and d).



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781 Figure 2. Boxplots for size rank of standard length and dry weight of each classified
782 developmental stage in Atlantic bluefin tuna and Atlantic bonito larvae. Different letters
783 indicate significant different means among stages (ANOVA, Tukey HSD, $p < 0.05$).

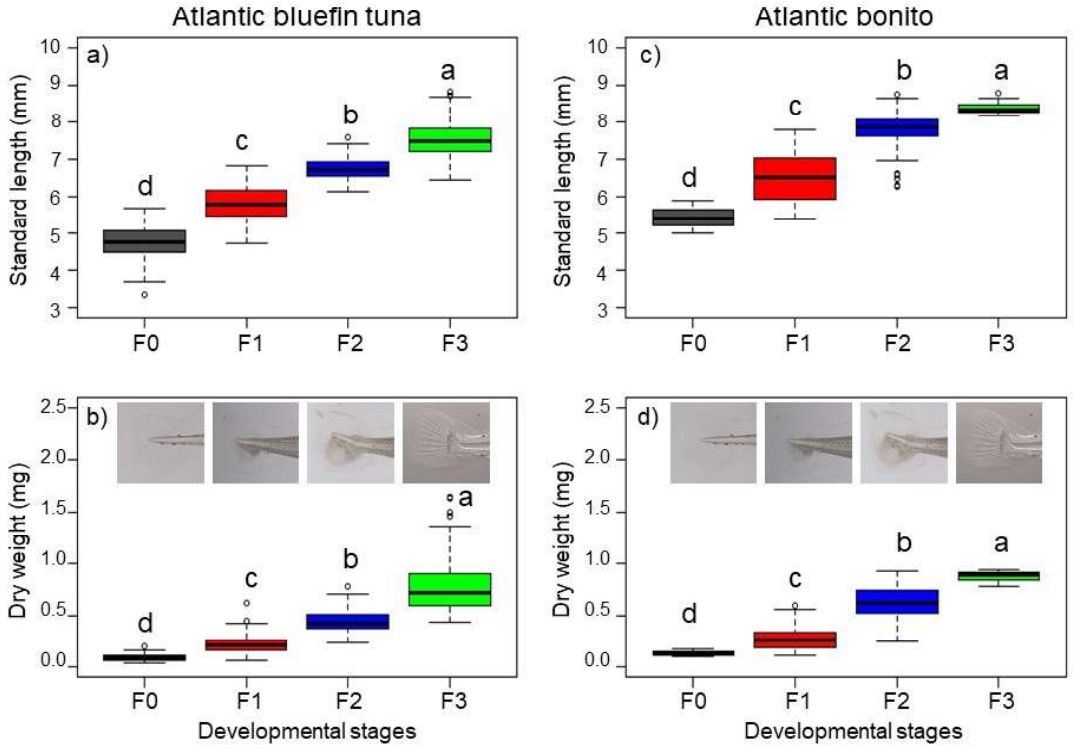

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Figure 3. Cumulative developmental stage proportion distributions showing the duration of the different developmental stages during the a) Atlantic bluefin tuna and b) Atlantic bonito ages. F0: larvae in pre-flexion, F1: larvae with development of the first caudal fin rays, F2: larvae in flexion and F3: larvae in post-flexion.

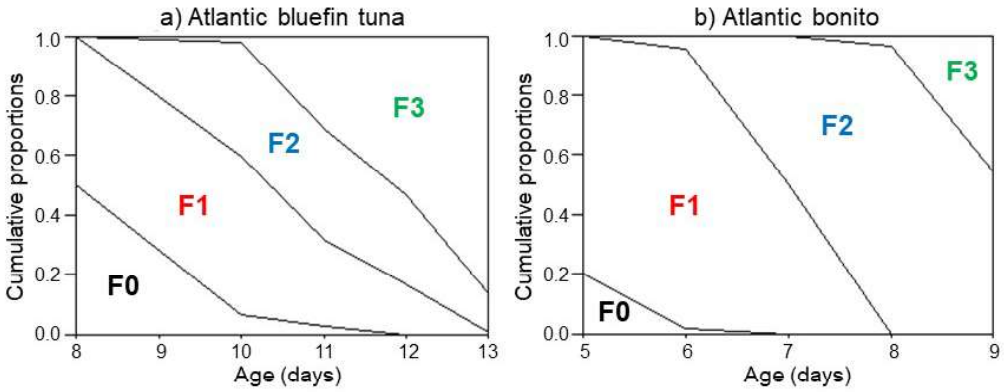
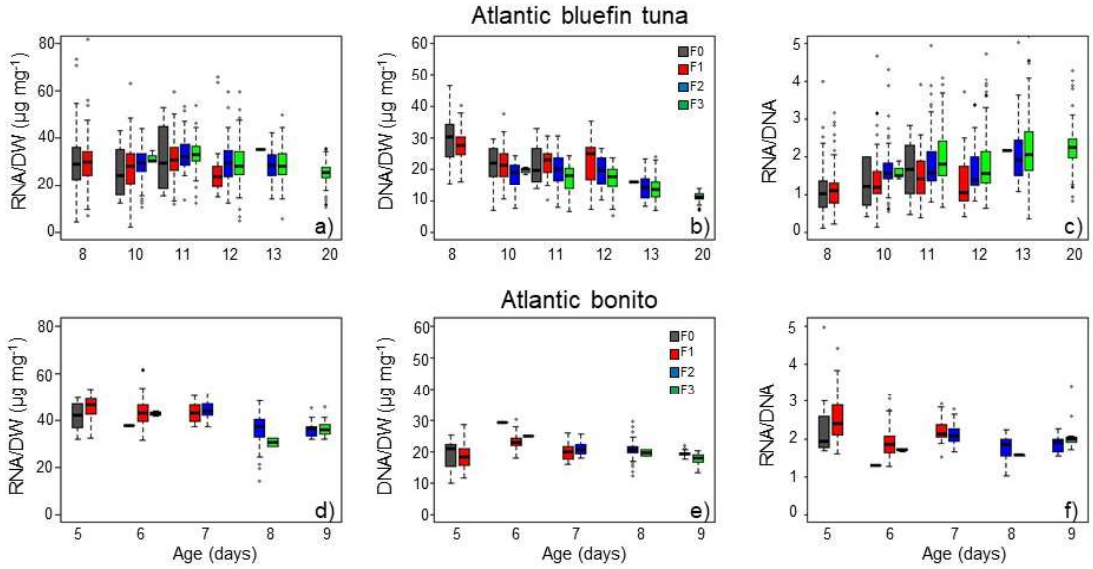


Figure 4. Boxplots of the daily larval nucleic acid relative measures : RNA:DW (µg mg^{-1}), DNA:DW (µg mg^{-1}) and RNA:DNA ratios in Atlantic bluefin tuna (a–c) and Atlantic bonito (d–f). Different stages are shown in different colors. Atlantic bluefin tuna larvae were sampled from 8 to 20 dph, while Atlantic bonito larvae were sampled from 5 to 9 dph.



808 Figure 5. DW-DPH residuals plotted against DNA-DW residuals in Atlantic bluefin tuna
809 larvae. Different stages are shown with different colors: F0: black, F1: red, F2: blue and
810 F3: green. A significant relationship was found in F0 stage (black line): $y = -0.419 + 0.566 \cdot x$, $R^2=0.122$, $p<0.01$, $n=90$ and F3 stage (green line): $y = 0.231 + 0.278 \cdot x$,
812 $R^2=0.056$, $p<0.01$, $n=270$.

813

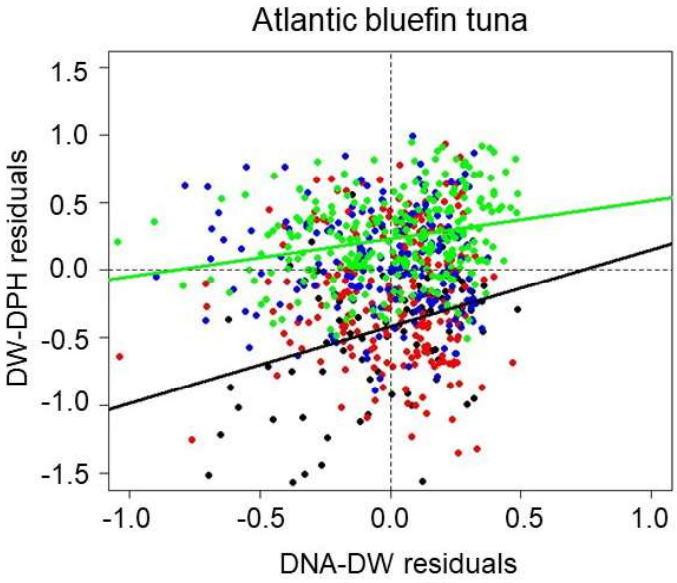


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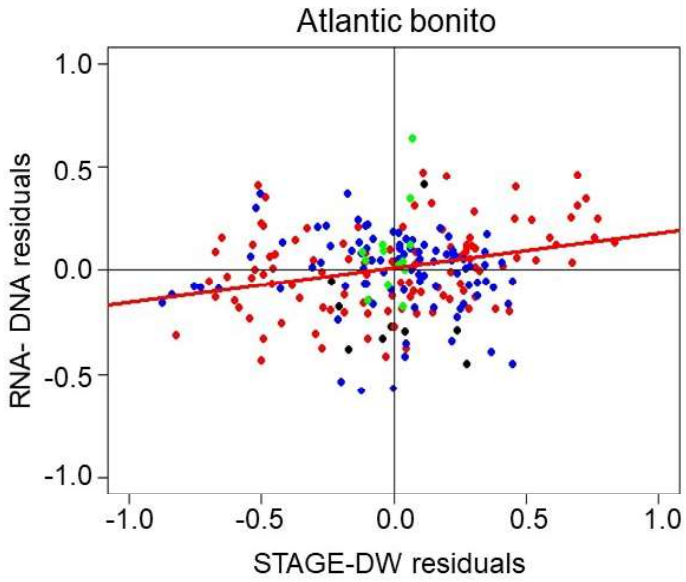
Figure 6. RNA-DNA residuals plotted against STAGE-DW residuals in Atlantic bonito larvae. Different stages are shown with different colors, F0: black, F1: red, F2: blue and F3: green. A significant relationship was found in F1 stage (black line): $y = 0.012 + 0.167 \cdot x$), R^2=0.102, p<0.001, n=115).

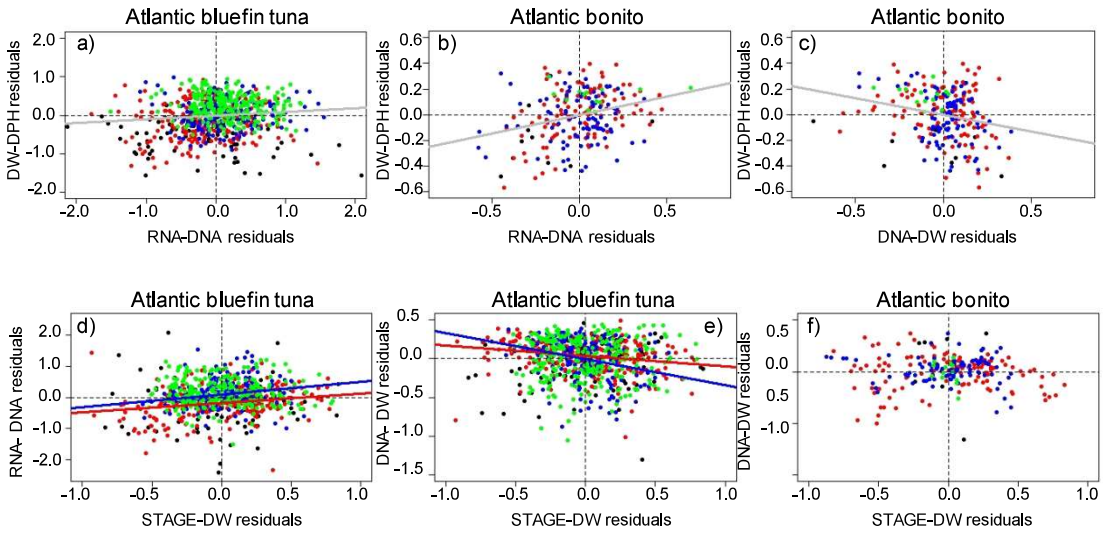


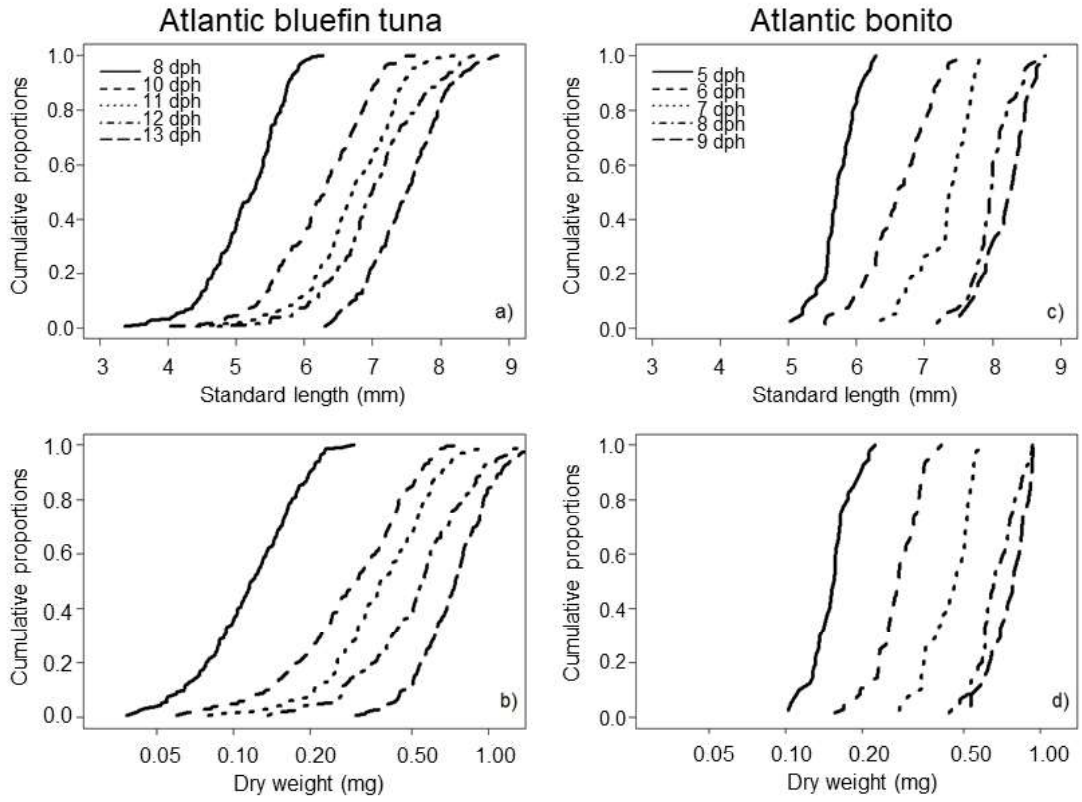
Supplementary material from "The effect of nutritional condition by two nucleic acid derived indices on the growth to post-flexion of Atlantic bluefin tuna and Atlantic bonito larvae"

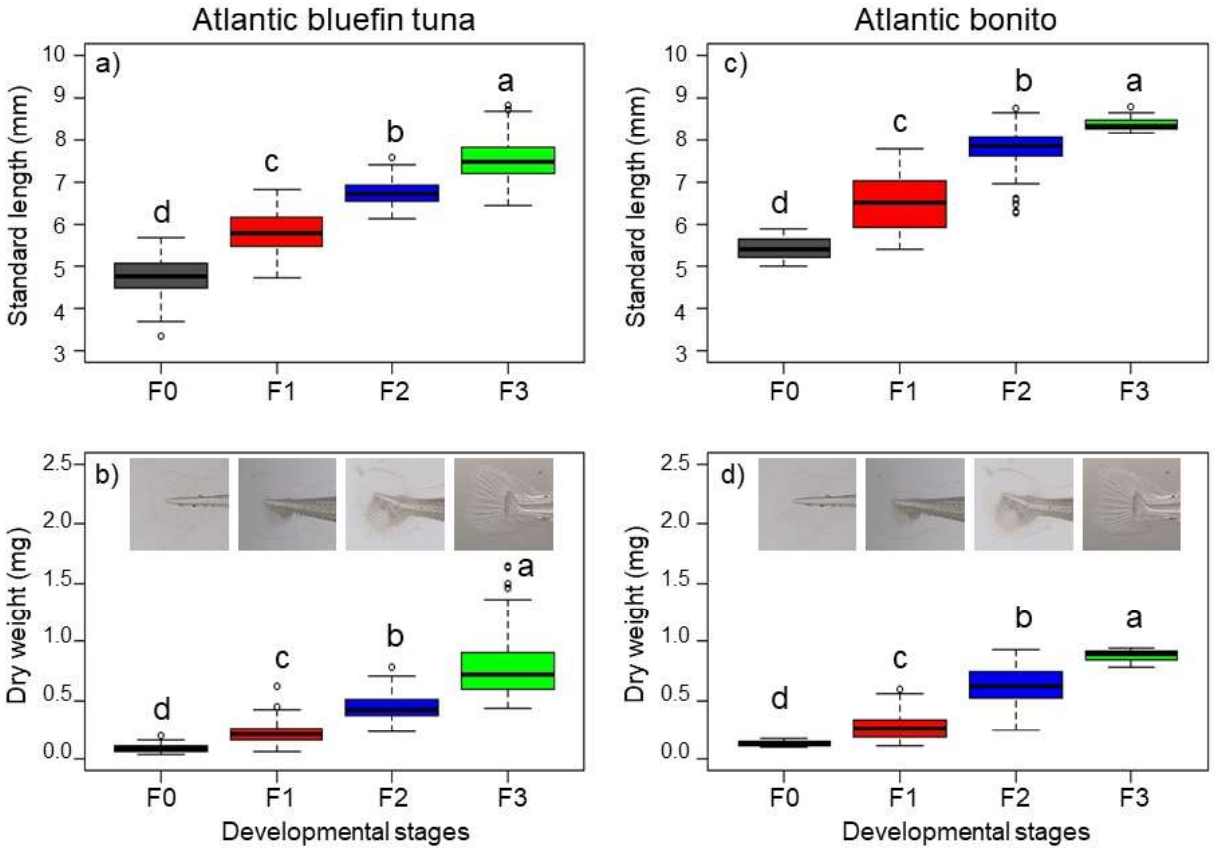
JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

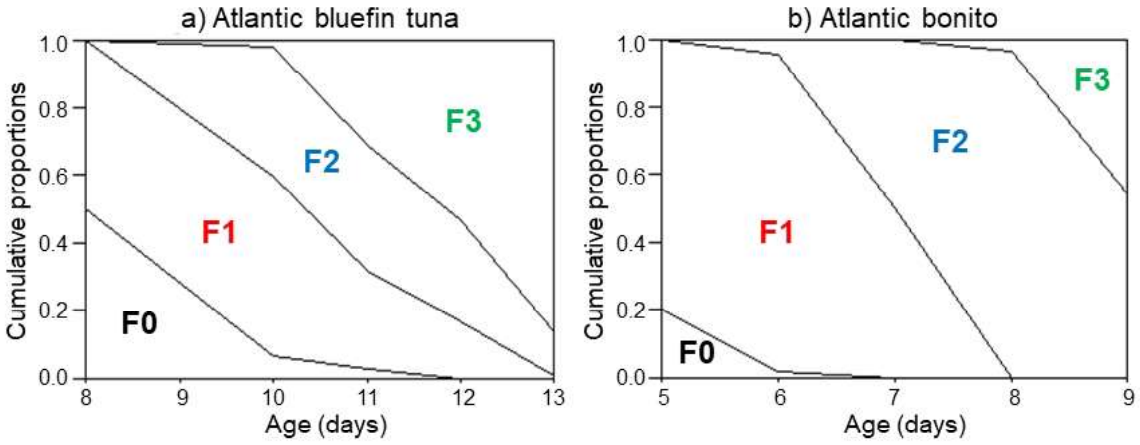
Authors: Edurne Blanco, Patricia Reglero, Alma Hernández de Rojas, Aurelio Ortega, Fernando de la Gándara, Arild Folkvord

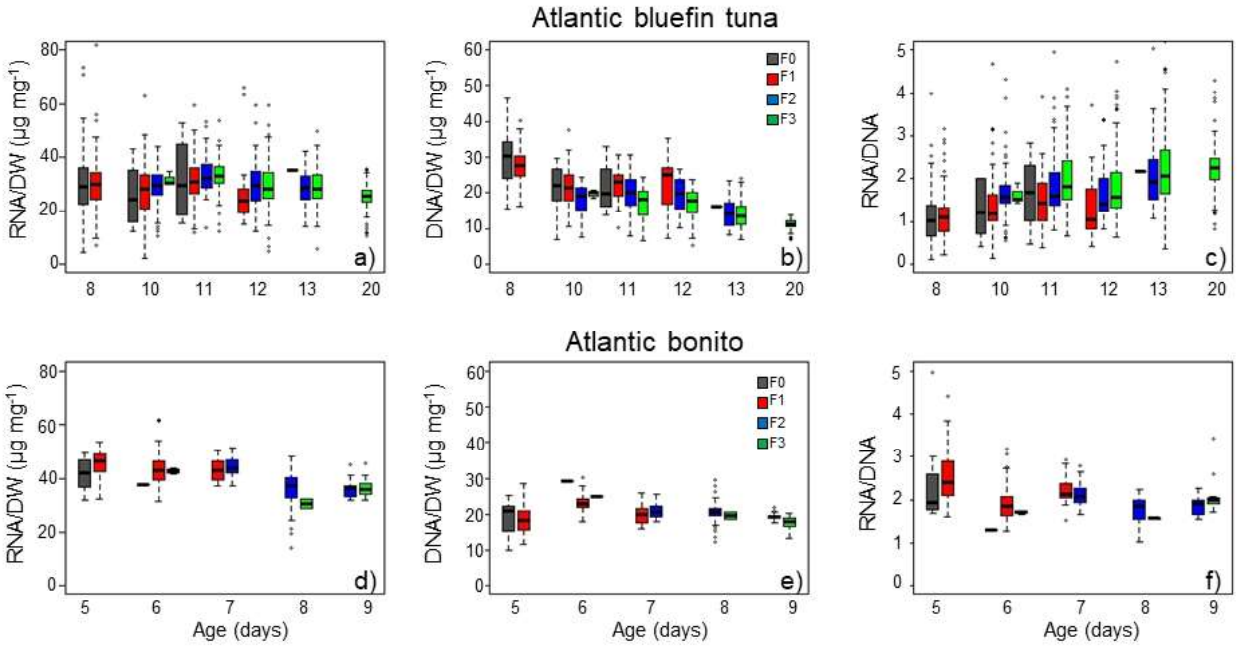
Supplementary Figure 1. RNA-DNA residuals and DNA-DW residuals plotted against DW-DPH (size-at-age) and STAGE-DW residuals (stage-at-size) in Atlantic bonito and Atlantic bluefin tuna larvae. Different stages are shown with different colors, F0: black, F1: red, F2: blue and F3: green. Significant relationship within stages are shown with color lines. The average relationship is represented by the grey line in those where no significant differences were found within stages.

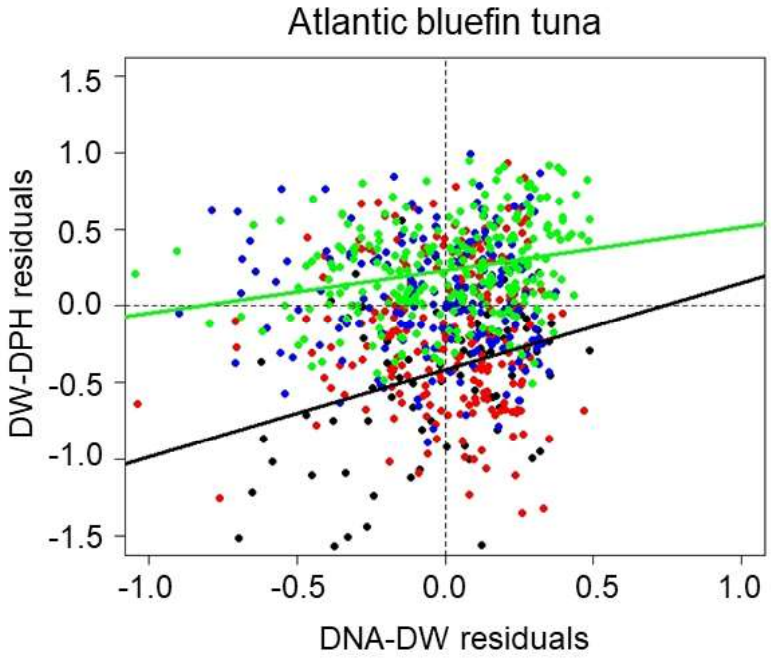


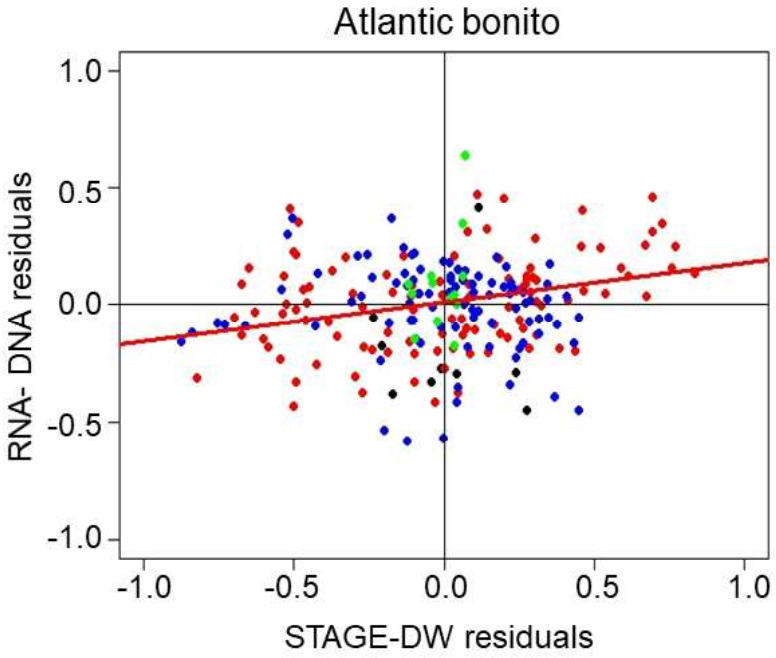

Atlantic bluefin tuna
Atlantic bonito
8 dph
10 dph
11 dph
12 dph
13 dph
5 dph
6 dph
7 dph
8 dph
9 dph
Cumulative proportions
Standard length (mm)
Dry weight (mg)
a)
b)
c)
d)

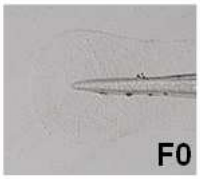
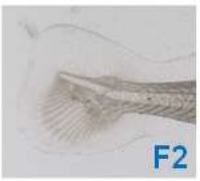

Atlantic bluefin tuna
Atlantic bonito
a)
b)
c)
d)
Standard length (mm)
Dry weight (mg)
F0
F1
F2
F3
Developmental stages
a
b
c
d

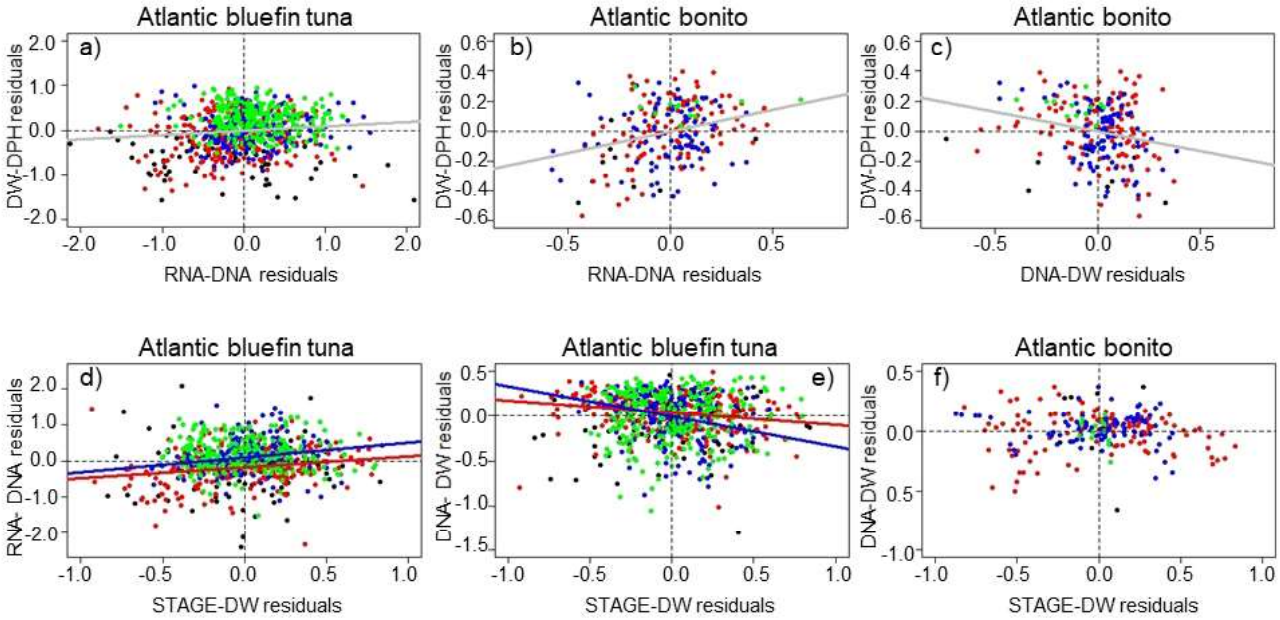

a) Atlantic bluefin tuna
Cumulative proportions
1.0
0.8
0.6
0.4
0.2
0.0
8
9
10
11
12
13
Age (days)
F0
F1
F2
F3
b) Atlantic bonito
Cumulative proportions
1.0
0.8
0.6
0.4
0.2
0.0
5
6
7
8
9
Age (days)
F0
F1
F2
F3


Atlantic bluefin tuna
RNA/DW (µg mg-1)
DNA/DW (µg mg-1)
RNA/DNA
F0
F1
F2
F3
a)
b)
c)
Atlantic bonito
d)
e)
f)
Age (days)


Atlantic bluefin tuna
DW-DPH residuals
DNA-DW residuals
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0


Atlantic bonito
RNA- DNA residuals
STAGE-DW residuals
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0

Stage	Nomenclature	Description	Example
Pre-flexion	F0	Straight notochord	
First caudal fin rays	F1	Straight notochord with some rays in the ventral side	
Flexion	F2	Bending upward of the notochord tip in a very clear angle with an increase in the amount of fin rays	
Post-flexion	F3	The final tip of the notochord disappears. Definition of the hypural plate and caudal fork. The posterior margin of the upper hypural plate is at 90 ° from the notochord axis	


Atlantic bluefin tuna
a)
DW-DPH residuals
RNA-DNA residuals
Atlantic bonito
b)
DW-DPH residuals
RNA-DNA residuals
Atlantic bonito
c)
DW-DPH residuals
DNA-DW residuals
Atlantic bluefin tuna
d)
RNA-DNA residuals
STAGE-DW residuals
Atlantic bluefin tuna
e)
DNA-DW residuals
STAGE-DW residuals
Atlantic bonito
f)
DNA-DW residuals
STAGE-DW residuals