EFFECT OF FOOD TYPE AND CONCENTRATION ON GROWTH AND FATTY ACID COMPOSITION OF EARLY LARVAE OF THE ANCHOVY (*Engraulis encrasicolus)* REARED UNDER LABORATORY CONDITIONS

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ABSTRACT

Experiments were conducted during the summer of 2008 and 2009 to study the growth of early post yolk-sac European anchovy (*Engraulis encrasicolus*, (Linnaeus, 1758) larvae reared under different food regimes. The fatty acid composition was used to assess nutritional condition of the larvae. Prey items used in the experiments were *Gymnodinium sanguineum*, *Brachionus plicatilis* and nauplii of the copepods *Acartia grani* and *Euterpina acutifrons*. Food type and concentration affected the growth of the larvae. Mixed diets composed of rotifers and copepod nauplii at high concentration resulted in higher anchovy larvae growth rates in comparison with single-prey diets using either rotifers or copepod nauplii. The addition of the dinoflagellate *Gymnodinium sanguineum* (25-50 cells.ml^{-1}) to the prey offered did not enhance significantly larval growth. Highest growth rates of anchovy larvae (0.28 mm d^{-1}) were obtained using high concentrations of a mixed diet, particularly the combination of rotifers and *Acartia grani* nauplii. Fatty acid composition at hatch was similar to the composition observed in the field, but during larvae ontogeny there was a marked decrease in the contribution of polyunsaturated fatty acids such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). Such difference reflects the high requirements of these PUFA for larvae development, and suggests that the food offered failed to fulfill the larvae nutritional requirements. The growth rates obtained in our experiments were, overall, in the lower range of those observed in natural conditions. Taking into considerations the fact that larvae in the field are expected to encounter lower prey concentrations, we discuss the reasons for such disagreement.

Keywords: Anchovy, *Engraulis encrasicolus*, fatty acids, larval growth

INTRODUCTION

Hjort (1926) proposed the hypothesis that year-class strength of marine pelagic fishes is determined within the first year of life, as a consequence of the high vulnerability of the larvae, which are susceptible of experiencing high mortalities. Such mortality seems to be related to inadequate feeding conditions at the time the yolk sac reserves are exhausted. Even if larvae don't die immediately, the slow growers are more susceptible to predation and become too weak to be able to recover, which is called the "point-of-no-return" (Blaxter and Hempel 1963). Other factors that significantly affect larval mortality are offshore advection and/or disruption of food patches that increase larval mortality due to starvation, past the first feeding phase (Wilhelm et al. 2005).

The European anchovy (*Engraulis encrasicolus*) is a pelagic fish distributed in the Mediterranean Sea and along the eastern Atlantic coast, from Norway to South Africa and represents one of the main targets of purse-seine fisheries for several countries located at Southern Europe and western Africa (Whitehead *et al.* 1988; ICES 2008). These fisheries have typically a large variability in population size, which is mostly attributed to recruitment variability. It is thought that variations in the recruitment strength of small pelagic fish are primarily driven by biological and/or physical factors impacting on early life stages (Mullon *et al.* 2003). In particular for the European anchovy, Aldanondo *et al.* (2010) demonstrated that larval survival during peak spawning seems to control the annual recruitment of this species in the Bay of Biscay. For this reason, coupled biological-physical models developed to explore the complex interactions between physical oceanography and fish recruitment have mainly focused on the early life history stages (review in Gallego *et al.* 2007). Such models must be parameterized with the vital rates (growth and development) of fish early-life in order to

75 properly mimic the larvae response under different environmental circumstances.
76 Although the growth rates of the European anchovy larvae have been extensively
77 studied through otolith microstructure analysis of individuals collected in the main areas
78 of distribution such as the Bay of Biscay (e.g. Cotano *et al.* 2008), several Portuguese
79 estuaries (e.g. Ré 1996; Ribeiro 1991), the Mediterranean Sea (e.g. Palomera *et al.*
80 1988; Catalan *et al.* 2010) and the Adriatic Sea (e.g. Regner and Dulcic 1990; Dulcic
81 1997), to our knowledge, no laboratory experiments have been conducted to study the
82 relation between prey availability and European anchovy larval growth. Due to this lack
83 of knowledge, up to date models developed to study this species have either described
84 the larvae as inert particles (e.g. Allain *et al.* 2007) or used the data available for related
85 species (Urtizberea *et al.* 2008; Politikos *et al.* 2011). These alternatives may introduce
86 strong biases that can only be solved by using species-specific bioenergetic parameters.
87 European anchovy larvae are mainly diurnal feeders (Ré 1996; Tudela *et al.* 2002). The
88 study of its diet in the Mediterranenan Sea (Tudela *et al.* 2002) shows it is mainly
89 composed of copepod eggs, nauplii and copepodites. Nauplii appears to be important
90 mainly for larvae <4mm, whereas as larvae grow copepodites gain importance,
91 reflecting the broader prey range through ontogeny. There is still, however, some
92 controversy regarding its diet in the natural environment. Morote *et al.* (2010) found for
93 anchovy larvae in the Mediterranean Sea that besides calanoid copepods, harpacticoids
94 (*Microsetella* and *Euterpina*) and cladocerans were significant contributors to the diet of
95 <9 mm larvae. Contrarily, Catalan *et al.* (2010) also in the Mediterranean Sea reported
96 that in all size-classes, larvae ingested essentially calanoid copepods and selected
97 negatively against cladocerans. Microzooplankton prey might be an important
98 component of the anchovy larvae diet, however this have not been properly quantified
99 in stomach content analysis due to rapid digestion and absence of hard remains. Rossi *et*

100 *al.* (2006) found a high percentage of 18:1(n-9) and 18:4(n-3) in small anchovy larvae
101 apparently reflecting feeding on prymnesiophytes, since those are usually enriched in
102 these fatty acids (Dalsgaard *et al.* 2003) and their abundance was high in the research
103 area.
104 There is a dramatic change in the ability to withstand starvation over the larval period,
105 with larvae being usually more vulnerable at first-feeding (Hunter 1976). Lipid
106 utilization in marine fish mainly occurs after hatching, reflecting the greater energy
107 demand of the free-swimming yolk-sac larvae compared to the egg (Sargent *et al.*
108 2002). The large demand of monounsaturated and polyunsaturated fatty acids for the
109 diet of the larvae at these critical early stages makes them crucial for their growth and
110 survival (Tocher 2003).
111 The objective of this work is two-fold: firstly, to determine, for the first time for this
112 species, laboratory-derived growth rates of first feeding anchovy larvae in relation to
113 different prey types and concentrations, and secondly, to study the variations of the fatty
114 acid composition of early larvae through ontogeny and in relation to their diet. Finally,
115 we will discuss our results in relation with the available field data on growth and fatty
116 acid composition of anchovy larvae.
117
118 **MATERIAL AND METHODS**
119
120 Two series of anchovy larvae experiments were done; the first during the summer of
121 2008 and the second during the summer of 2009 (Table I). For both series, anchovy
122 eggs were obtained from adult fish captured as juveniles by purse-seine fishery in
123 September of 2007 in the Bay of Biscay and maintained in the San Sebastian Aquarium
124 in cylindrical tanks (1300 L). Adult fish started spawning naturally using photoperiod,

125 temperature and increased food concentration stimulus. Each morning the egg collector
126 placed in the overflow of the adult fish tank was inspected, and when present, the eggs
127 were collected, counted and transferred by pipette into the experimental tanks.
128 Growth experiments were conducted in cylindrical containers wrapped with black
129 plastic, filled with 5-liter filtered seawater and kept in a temperature-controlled, air-
130 conditioned room. 5-L volumes were chosen to conduct the experiments because for the
131 first few weeks of larval life they have proven to offer as good conditions for larvae
132 growth as larger ones (Lasker *et al.* 1970), and they better allow to study quantitatively
133 early larval feeding. Salinity was maintained at 35.5 (PSS) while temperature was
134 maintained at 19-20ºC, corresponding to the sea temperature at which anchovy present
135 high spawning activity and good larval growth. Photoperiod was kept at 16 hours light
136 and 8 hours dark. Temperature, oxygen, salinity and water quality parameters (nitrates,
137 ammonia) were measured daily.
138 Anchovy larvae were provided known concentrations of different food types, which
139 were maintained through the whole experimental period. Experiments lasted until there
140 were no larvae in the tank, as the result of sampling and natural death (Table I). Prey
141 were introduced in the tanks from day 4 post hatch onwards and comprised the
142 dinoflagellate *Gymnodinium sanguineum,* the rotifer *Brachionus plicatilis* and nauplii of
143 two copepod species: the calanoid *Acartia grani* and the harpacticoid *Euterpina*
144 *acutifrons*, both cultured for several years at the *Institut de Ciències del Mar* (CSIC,
145 Barcelona). The prey types and concentrations used are within the preferred prey items
146 and range of prey concentrations reported for anchovy larvae in the field, and
147 commonly used in previous experimental studies of the growth and survival of another
148 engraulid species, the northern anchovy *Engraulis mordax* (Lasker *et al.* 1970, Kramer
149 and Zweifel 1970, O'Connell and Raymond 1970, Theilacker and MacMaster 1971,

150 Hunter 1976, Theilacker 1987). The microalgae *Isochrisis galbana* was also added to
151 all experimental tanks because although too small to be preyed upon by anchovy larvae,
152 it provided food to the rotifers and copepod nauplii offered to the anchovy larvae. The
153 tanks were supplied with gentle air bubbling, which has been shown not to affect small
154 pelagic fish larvae (Soura and Jerde 1977), to ensure the microalgae were kept in
155 suspension. Each morning, after quantifying the food remaining in the tank from the
156 previous day, 25% of the tank water was renewed and then new food items were added
157 to the tank to obtain the desired concentrations. Stock cultures of both copepod nauplii
158 were kept in a mixture of *Tetraselmis suecica* and *Isochrisis galbana*. The rotifers used
159 as prey were fed *Isochrisis galbana*, a microalgae described as a enhancer of the fatty
160 acid content in rotifers, especially 22:6n-3 (Dhert et al. 2001 and references within).
161 Concentrations of dinoflagellates were counted using a Sedgwick-Rafter counting
162 chamber under an inverse microscope; rotifers and copepod nauplii were counted as 4
163 replicates of 1 ml subsample under a stereoscope microscope. Prey were measured at
164 the start of the set of experiments and their carbon content estimated using equations
165 given in Smayda (1978) for dinoflagellates, and van der Lingen (2002) for crustacean
166 nauplii.
167 The anchovy larvae growth rate was assessed by sampling 5-10 larvae every 3 days and
168 measuring their total length. Length data were fitted to exponential growth curves:
169
170 $$SL - lo\, e^{kt}$$
171
172 where l_0 is length at hatching, k is the instantaneous growth rate and t is the age.
173 For the 3 experiments carried out during 2009 the fatty acid content of the zooplankton
174 prey, the anchovy eggs and the anchovy larvae were also analyzed (Table I). For that

purpose, 25-300 anchovy eggs were sampled at the beginning of the experiment, whereas for zooplankton, ca. 30 copepod nauplii and ca. 100000 rotifers were collected at the first day of larvae feeding; in addition, 10 anchovy larvae were sampled every 3 days until the end of the experiment for fatty acid analysis. The above-mentioned samples for fatty acid analysis were in all cases rinsed in distilled water and preserved in liquid nitrogen until analysis. After lyophilisation, lipid extraction and conversion of acyl groups into methyl ester derivatives (FAMES) was performed after Peters et al. (2007) using modified protocols of Folch et al. (1957) and Kattner and Fricke (1986). FAMES and fatty alcohols were separated by gas chromatography, detected by flame ionisation and identified by comparing retention times with those derived from standards of known composition.

All statistical analyses and data manipulations were performed using the open source software R version 2.9.2 (R Development Core Team 2009).

RESULTS

1 - Anchovy larvae growth

Anchovy larvae hatched approximately 48 h after collection of the eggs, measuring 2.71±0.74 (SD) mm. Instantaneous growth rates varied with the feeding level (Fig. 1 and Table I), ranging from 0.044 d^{-1} (in one of the experiments where larvae were fed rotifers as single prey) to 0.066 d^{-1} (in one experiment using a mixed diet of rotifers and *Acartia grani* nauplii). These instantaneous growth rates when computed as length growth for the first 15 days after hatch correspond to rates of 0.17 mm d^{-1} and 0.31 mm d^{-1}, respectively. In general, anchovy larvae growing with a diet that included rotifers and copepod nauplii grew faster than those fed rotifers as single prey, even if provided

200 in higher concentrations. Growth of anchovy larvae fed with a single diet of high
201 concentrations of *A. grani* (4 ind ml^{-1}) was also low and similar to that obtained using
202 rotifers alone. The addition of the dinoflagellate *Gymnodinium sanguineum* in the diet at
203 concentrations of 25-50 cells ml^{-1} did not enhance significantly first-feeding anchovy
204 larvae growth when compared to a diet of rotifers and copepod nauplii without
205 dinoflagellates. The mixed diet composed of *G. sanguineum*, rotifers and *Euterpina*
206 *acutifrons* nauplii resulted in lower growth rates (0.046 d^{-1}) than those obtained using
207 the same concentration of the nauplii of the calanoid copepod *A. grani* (0.048 d^{-1}).
208 The covariance analysis relating larvae size with larval age and experiment (as factors)
209 was significant ($p<0.0001$, $R^2=73\%$) and showed that overall the growth rates obtained
210 among experiments were not significantly different from each other except for
211 experiment #8 ($p=0.05$) and experiment #11 ($p=0.03$), which resulted in significantly
212 higher growth rates (Fig. 2). Those experiments corresponded to larvae fed a mixture of
213 rotifers and *A. grani* nauplii (1 ind ml^{-1}, each), and in case of experiment #11 the
214 dinoflagellate *Gymnodinium* had also been added to the diet.
215 The instantaneous growth rate was not linearly related to the total prey carbon content
216 of the diet, and this lack of relationship was caused by the effect of prey composition on
217 larval growth rates (Fig. 3). Indeed, experiment #6 had the highest carbon content diet
218 (composed of 4 acartia nauplii ml^{-1}) but resulted in low larval growth rates. Despite all
219 the other experiments had similar total carbon content diets, experiment #8 in which
220 copepod nauplii (1 ind ml^{-1}) were combined with a low concentration of 1 rotifer ml^{-1}
221 resulted in the highest growth rates observed in our study.
222
223 **2 – Fatty acid composition of prey, eggs and larvae**
224

In the experiments conducted during 2009, the fatty acid composition of the zooplankton prey, eggs and anchovy larvae was determined (Table I). The fatty acid composition of the eggs did not vary significantly among eggs spawned at different days of the 2009 spawning season (Fig. 4). The fatty acid 16:0 had the highest contribution to the total fatty acid content of anchovy eggs (30.8±1.16 % of total fatty acids (tFA)) followed by 22:6(n-3) or docosahexaenoic acid (DHA, 24.0±1.35% tFA) and the monounsaturated 18:1(n-9) (13.8±0.39% tFA, respectively). The FA 20:5(n-3) or eicosapentaenoic acid (EPA) represented 7.7±0.28% tFA of the total egg fatty acid content (Fig. 4).

Both copepod nauplii species had a higher percentage of polyunsaturated fatty acids than the rotifers, including EPA and DHA (Fig. 5). *A. grani* nauplii had a higher percentage of 20:5(n.3) and 18:1(n-9) whereas *E. acutifrons* nauplii had a higher 22:6(n-3) content. Rotifers, on the other hand, presented higher proportion of monounsaturated fatty acids, particularly of 16:1(n-7) and 18:1(n-9) (Fig. 5).

Regarding the anchovy larvae, of the 17 fatty acids identified the most abundant were the 16:0 and 22:6(n-3), followed by the 18:1(n-9) and 18:0 and finally 20:5(n-3), (Table II). Overall, polyunsaturated fatty acids were the most relevant fatty acid group in all the larvae analyzed. However the percentage of (n-3) PUFA, particularly EPA and DHA, decreased significantly through ontogeny until day 5, when the yolk sac was being used (Fig. 6). After day 5 the decreasing trend of polyunsaturated fatty acids continued while some uptake occurred in two experiments, after 5dph for experiment 11 and after 11 dph for experiment 10 (Fig. 6).

DISCUSSION

As expected, we observed an effect of prey concentration and prey type on larval anchovy growth. Larvae grew the fastest with a mixed diet of rotifers and copepod nauplii, with concentrations of 1 prey ml^{-1} respectively. Diets using higher concentrations of either rotifers or acartia nauplii as single prey resulted in lower growth rates than the mixed diets tested. Just after the beginning of exogenous feeding, which occurred 3-4 days after hatch at 19ºC, anchovy larvae probably depended mostly of prey which were smaller and less motile than copepod nauplii, and only a few days later, when already larger, larvae were able to pursue and capture the copepod nauplii efficiently. Indeed, the inclusion of rotifers in the mixed diet enhanced anchovy growth, whereas high concentrations of *Acartia grani* nauplii supplied as single prey were not sufficient to sustain high growth rates for first feeding larvae. These observations are in agreement with general patterns of mouth size - prey size relationships in fish larvae (Sabates & Saiz 2000), in which small mouths (gap size) restrict not only the size of prey but also the range of prey sizes. Specifically for anchovy larvae, Morote ***et al.*** (2010) already found this restriction in early stages the European anchovy; similarly, the northern anchovy larvae appeared to fail to capture copepod nauplii (*Tigriopus* sp.) at 1 ml^{-1} until reaching 7-8 dph (Theilacker 1987).

The comparison of the growth rates of anchovy larvae in those experiments fed, at the same nauplii density, either of both copepod species nauplii shows that *Acartia grani* nauplii provided higher growth rates than the nauplii of the harpacticoid *Euterpina acutifrons*. Such difference agrees with several field studies showing that calanoid copepods are preferentially selected in comparison with the harpacticoid ones, therefore constituting the bulk of the anchovy larvae's diet (Conway 1998; Catalan ***et al.*** 2010). We expected that the addition of the dinoflagellate *Gymnodinium splendens,* which falls into the same size range as small rotifers but are generally considered more nutritional,

275 especially in terms of fatty acid content (Mansour *et al.* 1997), to larval diet would
276 enhance anchovy larvae growth, but it did not. This result contrasts with the
277 observations of Lasker *et al.* (1970), who were able to grow first feeding northern
278 anchovy larvae on a single diet of the dinoflagellates *Gymnodinium splendens* on
279 slightly higher concentrations. Probably this lack of conspicuous effect in our
280 experiments was due to the use of moderate to high densities (25-50 cells ml^{-1}) of the
281 dinoflagellate. Further efforts have to be devoted to identify the actual small prey that
282 provides suitable food to sustain high growth and survival at those first days of
283 exogenous feeding, so critical for the larvae survival; ciliates and dinoflagellates are
284 good candidates (Scura and Jerde 1977; Nagano *et al.* 2000).
285 The growth rates we have obtained in the laboratory for the European anchovy larvae
286 are comparable, at high prey concentrations, to the growth rates obtained in the
287 laboratory for the northern anchovy (Theilacker, 1987; 0.31 mm d^{-1} and 0.33 mm d^{-1} at
288 15 dph, respectively, for the European anchovy and the northern anchovy larvae) and
289 lower than those obtained for first-feeding japanese anchovy, E. japonica (Mito et al.,
290 1973, Fukuhara, 1983; 0.57 mm d^{-1} and 0.43 mm d^{-1}, respectively). This is in agreement
291 with the fact that the prey types and concentrations used in our experiments were similar
292 to those used in previous experiments carried out with the northern anchovy larvae and
293 lower than the feeding concentrations used for Japanese anchovies. The maximum
294 growth rate obtained in the experiments using a combination of rotifers and *A. grani*
295 nauplii is slightly higher than the growth rate determined in previous experiment using
296 rotifers *ad libitum* (Aldanondo *et al.* 2008). The growth rates obtained here were also
297 slightly higher than those estimated for first-feeding larvae of the same species (known
298 as *Engraulis capensis* when the work was done), in experiments conducted in South
299 Africa (Brownell, 1983) using unknown concentrations of calanoid and cyclopoid

300 copepods until 15 dph, and rotifers this day onwards. The comparison of our lab-
301 determined growth rates with field data (estimated from otolith microstructure analysis
302 of field caught larvae, Fig. 7) evidences a disagreement, our rates being at the lower
303 range of field values (field data include growth rates obtained in the wild for larvae
304 growing with different food concentrations, temperatures and salinities, explaining their
305 high variability). Growth rates obtained in our experiments ranged between 0.17 and
306 0.31 mm d^{-1} for larvae growing 15 dph, contrasting with the mean growth rates
307 estimated for the larvae caught in the Bay of Biscay (0.4-0.6 mm d^{-1}, Cotano *et al.*
308 2008), several Portuguese estuaries (0.25-0.51 mm d^{-1}, Ré 1996; Ribeiro 1991), in the
309 Mediterranean Sea (0.4 - 0.91 mm d^{-1}, Garcia *et al.* 1998; Palomera *et al.* 1988; Sabates
310 *et al.* 2007; Somarakis *et al.* 2007; Catalan *et al.* 2010) and the Adriatic Sea (0.9-0.94
311 mm d^{-1}, Regner and Dulcic 1990; Dulcic 1997). The difference between laboratory
312 experiments and field estimates might even be slightly larger because field estimates
313 have assumed that the initial increment deposition in the otoliths of *E. encrasicolus*
314 larvae occurs at the beginning of first feeding or 2 days pos hatch but recent laboratory
315 experimentation (Aldanondo *et al.* 2008) revealed that the first increment occurs on the
316 day after hatch, which means that previous growth rates are slightly underestimated,
317 especially for small larvae. The same discrepancy between laboratory-derived and field
318 estimates of larval growth rates were also observed for the Japanese anchovy, *Engraulis*
319 *japonica* (Mito et al., 1973, Takasuka et al. 2009), and other related species such as
320 European sardines (Blaxter, 1969) and Atlantic herring (Checkley Jr., 1984).
321 The fact that the larval anchovy growth rates obtained in laboratory conditions are in the
322 lower range of the natural variation can probably be explained by growth-limiting prey
323 concentrations, the use of unfitted prey type or size or larval stocking density. For
324 northern anchovy larvae lab-determined growth rates were closer to field values

325 (Methot and Krammer 1979) than for the European anchovy but no field larvae
326 presented growth rates as slow as the laboratory ones using limited rations (Methot and
327 Krammer 1979). This fact led the authors to presume the existence of growth-rate
328 dependent selective mortality in anchovy, especially if the mean growth rate is low and
329 the slower growing individuals are near starvation. On the other hand, the absence of
330 slow growers in the field can be explained by the presence of predators. In fact, for the
331 larvae of the Japanese anchovy *Engraulis japonicus*, it was shown that slow growing
332 larvae are more vulnerable to predation (Takasuka et al. 2003). Predation is probably
333 crucial for larvae survival in the field resulting in a growth dependent mortality as
334 opposed to larvae growing in the laboratory in the absence of predators. On the other
335 hand, the larval stocking density of the experiments was higher than the larval densities
336 found in the wild, which might have affected larval foraging behavior and competition
337 and resulted in lower growth rates than the expected from the high concentrations of
338 prey offered.
339 For the sake of the discussion, one may attempt at calculating the food intake necessary
340 to achieve such larvae growth rates. Using the relation between anchovy larvae size (*SL*,
341 mm) and dry weight (*DW*) estimated by Catalan *et al.* (2010):
342 $\ln DW = 0.867 * 0.5\ SL$
343 the increase in dry weight for a larvae growing in lenght from 7 to 8 mm is 51.1 µg,
344 which equals 20.45 µg C assuming that carbon content is 40% of dry weight. We
345 further take into consideration that 7-8 mm anchovy larvae feed mainly on calanoid
346 copepod nauplii, with modal prey size of 120 µm (Morote *et al.* 2010), which in terms
347 of biomass correspond to a prey with carbon content of 0.035 ng C ind^{-1} (Henriksen *et*
348 *al.* 2007). Assuming 40% growth efficiency, anchovy larvae should ingest ca. 580
349 nauplii daily in order to grow equivalently to the average field growth rate estimate, 0.4

mm d^{-1} (25.8% increase of larvae weight). Similarly, to achieve a growth similar to the maximum growth rates found in our laboratory experiments (24.4% increase in dry weight), ca. 200 nauplii should be ingested daily; this value rises up to ca. 1500 nauplii in order to be able to grow 1 mm d^{-1} (34% of larvae dry weight; maximum growth rate found the field, Cotano *et al.* 2008).

The high daily rations needed to sustain such field-observed growth rates imply that larvae must be feeding in copepod nauplii patches or else they would not have sufficient food to be able to survive. Conway *et al.* (1998) compared in the Adriatic Sea the abundance of potential prey items in the water column with the gut contents of anchovy larvae, and concluded that the abundance of potential prey coincident to anchovy larvae vertical distribution ranged between 0.018 and 0.2 prey items ml^{-1}, which is far lower than the concentrations used in our experiments. Similarly, Esteves *et al.* (2000) in an estuary located in the western Portuguese coast reported that the concentrations of copepod nauplii overlapping with the anchovy larvae distribution ranged from 0.05 to 0.6 individuals l^{-1}. The threshold concentrations of food required for survival of marine fish larvae in the laboratory are commonly much higher than the average prey concentrations found at sea, and more than 3 decades ago Hunter and Thomas (1974) argued that this disagreement must be related to the capability of fish larvae to find and remain in patches of food in the water column, with prey densities much higher than the average, integrated valued estimated from plankton net catches. At 6 mm anchovy larvae have visible swim bladders (Re´ 1996; Somarakis 2007) and vertical migrations initiate (Olivar *et al.* 2001), which has been proposed to be an important mark for the larvae as schooling behavior might begin during that phase (Somarakis *et al.* 2010), allowing them, at some extent, to move to areas of high food concentration. Anchovies spawn near or at the river estuaries, which indicates that the first larval stages must be

very dependent on the high productivity associated to those systems. In fact, in the Adriatic Sea the peak anchovy larvae concentrations were found in the immediate outflow area of the river flow, coincident with layers with high concentrations of potential prey items, typically reaching >0.5 prey l^{-1} (Coombs *et al.* 2003).

Fatty acids determined in anchovy larvae collected in the Mediterranean Sea (Rossi *et al.* 2006) presented similar proportion of fatty acids to the larvae analyzed in the experiments. However, these authors observed an exponential accumulation of polyunsaturated fatty acids (PUFA) through ontogeny, especially in the early stages of the anchovy development. This is contrary to the results of our experiments, in which the PUFA content of the larvae decreased as they grow, probably reflecting the inadequacy of the diet offered, that comprised *G. splendens*, rotifers and copepod nauplii. In our experiments, the n-3 PUFA were the fatty acids that presented the most rapid decline through early larval development, including the essential fatty acids DHA and EPA, which suggests that they are being utilized as energy substrate in the growing larvae. This utilization of n-3 highly unsaturated fatty acids as energy source for the developing larvae has been already reported for other fish species such as the white seabream (Cejas *et al.* 2004) and haddock (Plante *et al.* 2007), in which the decrease in PUFA content might occur naturally and is not necessarily the result of an inadequate, not nutritionally complete diet.

The proportion of 16:1(n-7) and EPA, that are biomarkers of diatoms, were low and similar to those estimated for early larvae (Rossi *et al.* 2006) and lower than the proportions determined for late larvae (Costalago *et al.* 2011), both collected off the Mediterranean Sea. When compared to other species, these diatom biomarkers are generally low for anchovy larvae, suggesting that diatoms are not a significant part of anchovy larval diet, which is confirmed by the low preference for diatoms observed at

sea (Costalago *et al.* 2011). However, EPA is an essential fatty acid for the developing larvae and must come from the diet. Fatty acid limitation is likely strongest in laboratory experiments where the zooplankton to serve as fish larvae is fed a monospecific diet (Anderson and Pond 2000). In future experiments diatoms, although not eaten directly by anchovy larvae, should be certainly supplied to the stock cultures of rotifers and copepods to be used for feeding fish larvae, in order to ensure sufficient amounts of EPA in the fish larvae diet that can eventually used for larvae development. In this study we have found that despite offering relatively high food concentrations, the obtained growth rates of first-feeding anchovy larvae were at the lower range of the natural variation. Since no individuals are found in nature with growth rates as low as those found under the restricted feeding conditions in our experiments, we can conclude that growth-dependent mortality at sea must play a major role in determining the recruitment success of the early stages of anchovy larvae. In addition, estimates of food consumption have shown that, in order to sustain the high growth rates found in nature, the early larvae must have very high daily rations. Single prey diets failed to support larvae growth, probably due to the rapidly changing range of preferred prey size through ontogeny. We expect that using higher concentrations of mixed diets including different prey sizes with adequate nutritional condition, particularly those containing high levels of polyunsaturated fatty acids, could enhance anchovy larvae growth to levels similar to those found under natural conditions. Such results would be valuable to parameterize current attempts to model anchovy larvae growth and dispersal. Moreover, the successful rearing of these larvae will enable a wide variety of further experimental studies to examine the impacts of abiotic factors on larval growth and survival.

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681 **Figure Captions**
682
683 **Fig. 1 –** Scatterplots showing larval size against age for 11 experiments where larvae
684 were fed different food types and concentrations; *Rot*: rotifers, *Ac*: *Acartia grani*
685 nauplii, *Eut*: *Euterpina acutifrons* nauplii, *Gym*: *Gymnodinium sanguineum.* Details of
686 the experiments are given in Table I.
687
688 **Fig. 2 –** Growth rates of first feeding anchovy larvae for experiment 8 *(dashed line,*
689 *dark circle);* experiment 11 *(dashed line, dark square)* and for the remaining
690 experiments (*solid line, open circles*). Details of the experiments are given in Table I.
691
692 **Fig. 3 –** Relation between the Instantaneous growth rate of first feeding anchovy larvae
693 and total prey carbon content (prey μgC ml^{-1}). The different diets used in the
694 experiments are represented: *rot: Brachionus plicatilis; ac: Acartia grani* naupli, eut:
695 *Euterpina acutifrons* nauplii, *gym: Gymnodinium sanguineum*.
696
697 **Figure 4** – Fatty acid composition (percentage of total fatty acids) of anchovy eggs
698 spawned in laboratory conditions during 2009. Results correspond to 6 pools of 100-300
699 eggs spawned during 2009.
700
701 **Figure 5 –** Fatty acid composition (percentage of total fatty acids) of the prey used to
702 feed anchovy first feeding larvae in the laboratory experiments of 2009.
703
704

705 **Figure 6** – Percentage of A) DHA and B) EPA content in relation to total fatty acid
706 content of the first feeding anchovy larvae of the growth experiments 9, 10 and 11
707 conducted during 2009. Details of the experiments are given in Table I.
708
709 **Figure 7 –** Growth of first-feeding anchovy larvae estimated from the laboratory
710 experiments (blank circles), conducted in the present work and in the experiments
711 conducted in Aldanondo *et al.*, 2008 (asterisk); and growth rates obtained from field
712 data (solid circles) off the Bay of Biscay, Western Portuguese coast and Mediterranean
713 and Adriatic Seas (Regner and Dulcic 1990, Ribeiro 1991, Ré 1996, Dulcic 1997,
714 Garcia *et al.* 1998, Palomera *et al.* 1988, Sabates *et al.* 2007, Somarakis *et al.* 2007,
715 Cotano *et al.* 2008, Catalan *et al.* 2010).
716

717 **Table I -** Conditions and results of the anchovy larvae growth experiments. *Rot*. Rotifers (*Brachionus plicatilis*), *A. grani* (*Acartia grani* nauplii),

718 *Gymno* (*Gymnodinium sanguineum*), *Euterp*. (*Euterpina acutifrons* nauplii).

	2008								2009		
	Exp. 1	Exp.2	Exp.3	Exp.4	Exp.5	Exp.6	Exp.7	Exp.8	Exp.9	Exp.10	Exp.11
Prey species	Rot.	Rot.	Rot.	Rot.	Rot.	A. grani	Rot. A. grani	Rot. A. grani	Gymno. Rot. A. grani	Gymno. Rot. Euterp.	Gymno. Rot. A. grani
[Prey] (n ml^{-1})	7	4	8	6	10	4	2.5 0.5	1 1	25 2.5 0.1	50 5 0.5	50 5 0.5
Initial density of eggs (n ml^{-1})	0.052	0.052	0.052	0.14	0.018	0.21	0.008	0.008	0.11	0.11	0.29
Starting day	3 Jul 08	3 Jul 08	3 Jul 08	20 Jul 08	3 Jul 08	5 Jul 08	17 Jul 08	17 Jul 08	23 Jul 09	23 Jul 09	26 Jul 09

Duration of experiments (dph)	9	12	15	9	24	8	12	12	8	11	14
Growth	x	x	x	x	x	x	x	x	x	x	x
Fatty acids of prey, eggs and larvae									x	x	x

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720

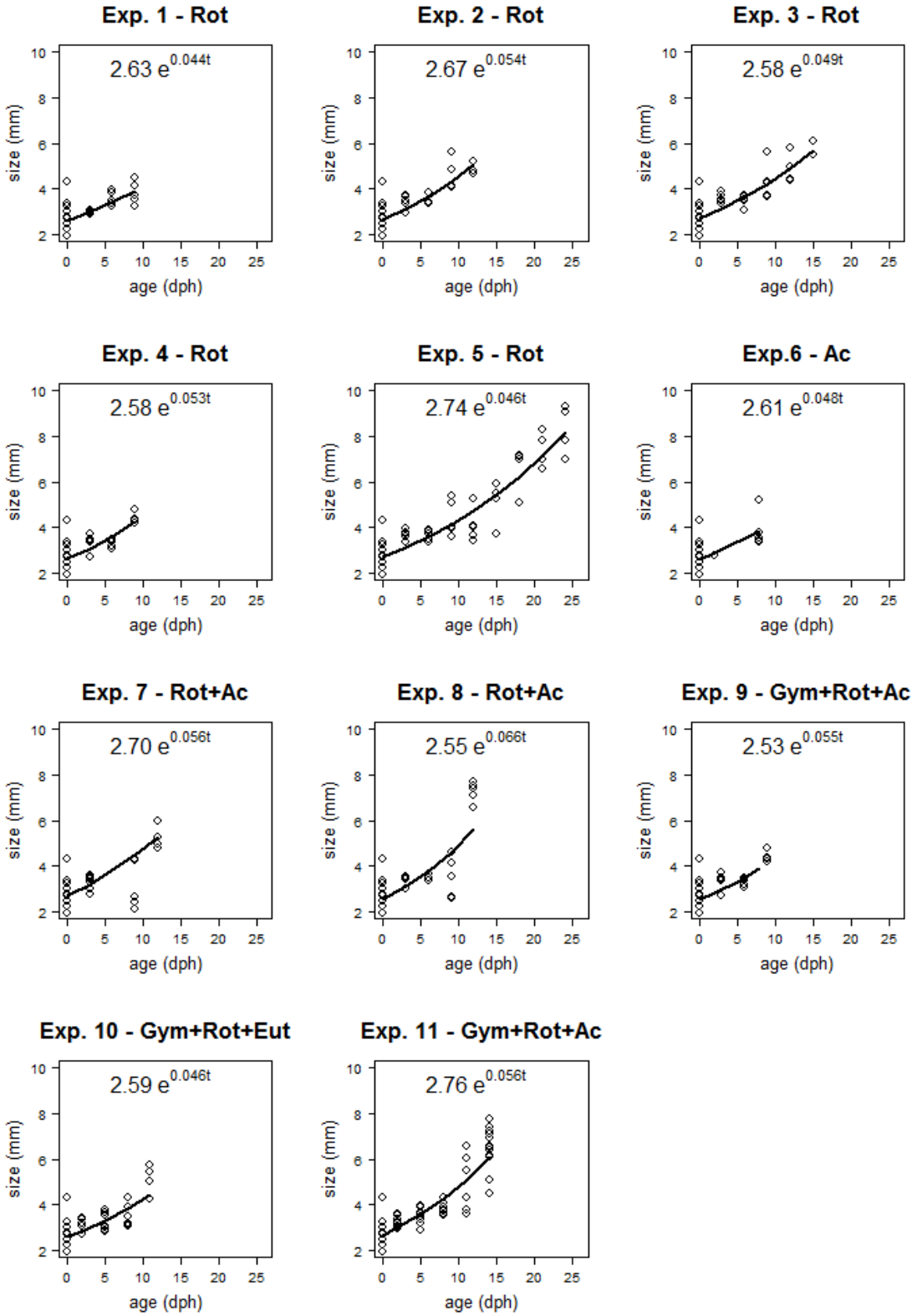
721

722 **Table II** – Fatty acid composition (percentage of total fatty acids) of the first feeding
723 anchovy larvae of the growth experiments conducted during 2009. Details of the
724 experiments are given in Table I.
725

Experiment		**9**			**10**				**11**				
Larvae age (dph)		**2**	**5**	**8**	**2**	**5**	**8**	**11**	**2**	**5**	**8**	**11**	**14**
%FA	**14:0**	1.2	2.0	0.0	1.2	1.8	7.9	1.3	1.1	2.7	1.2	2.5	5.2
	16:0	28.6	26.7	25.0	28.6	27.4	37.5	14.3	26.0	29.0	22.1	16.1	15.0
	18:0	8.1	11.3	13.0	8.1	12.1	13.6	12.1	8.9	13.6	14.8	11.1	9.2
	16:1(n-7)	2.2	3.1	2.4	2.2	2.1	2.6	7.5	1.7	2.0	2.8	7.0	11.7
	16:1(n-9)	1.1	4.0	0.0	1.1	2.7	1.4	2.1	0.0	1.8	1.7	2.4	1.9
	18:1(n-7)	3.3	2.7	3.5	3.3	2.7	2.1	10.5	3.6	3.0	3.6	8.4	8.8
	18:1(n-9)	11.6	12.1	10.7	11.6	10.9	9.6	14.5	13.7	10.4	10.5	13.3	14.9
	18:2(n-6)	5.0	3.9	4.0	5.0	3.5	1.1	3.0	4.3	3.2	3.9	3.5	3.0
	18:3(n-3)	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	1.9	2.1
	18:4(n-3)	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0	2.5	3.2
	20:5(n-3)	6.5	3.5	4.6	6.5	3.8	1.4	3.9	6.5	3.4	4.5	5.2	3.5
	22:5(n-3)	2.8	2.9	2.3	2.8	2.2	0.0	1.6	2.8	2.0	2.2	2.1	1.4
	22:6(n-3)	27.6	20.0	28.3	27.6	23.2	9.3	12.5	24.8	20.9	22.6	15.1	8.3

726
727
728
729

730 **FIGURE 1**



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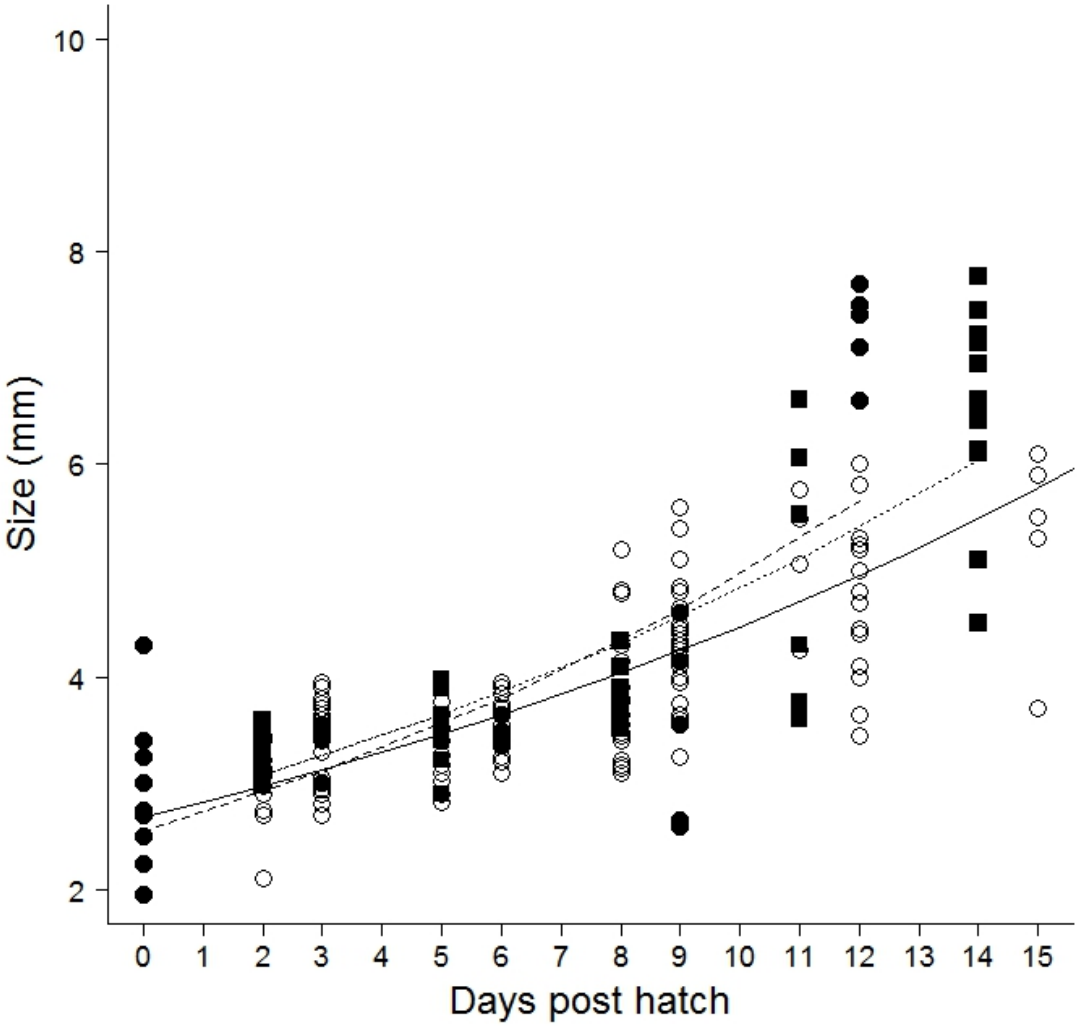
734 **FIGURE 2**

735

736



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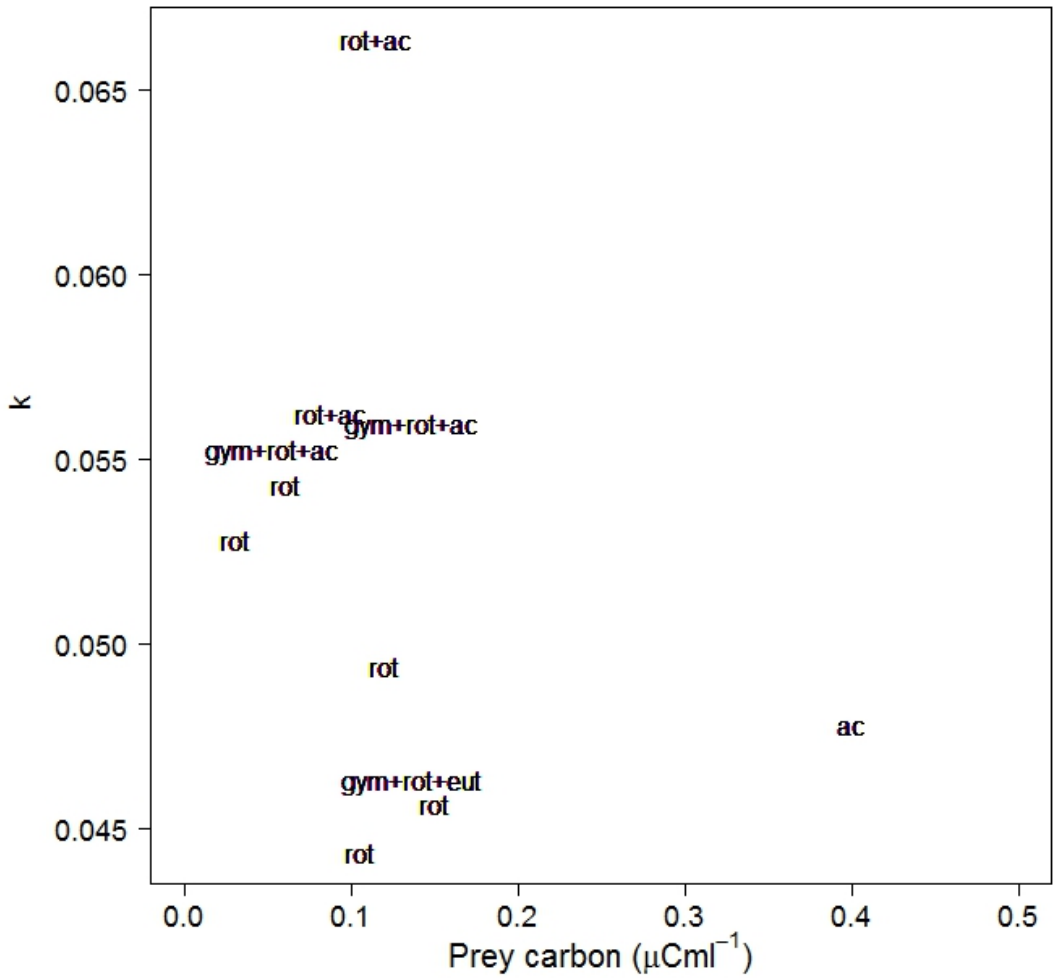
740 **FIGURE 3**

741



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744

745 **FIGURE 4**

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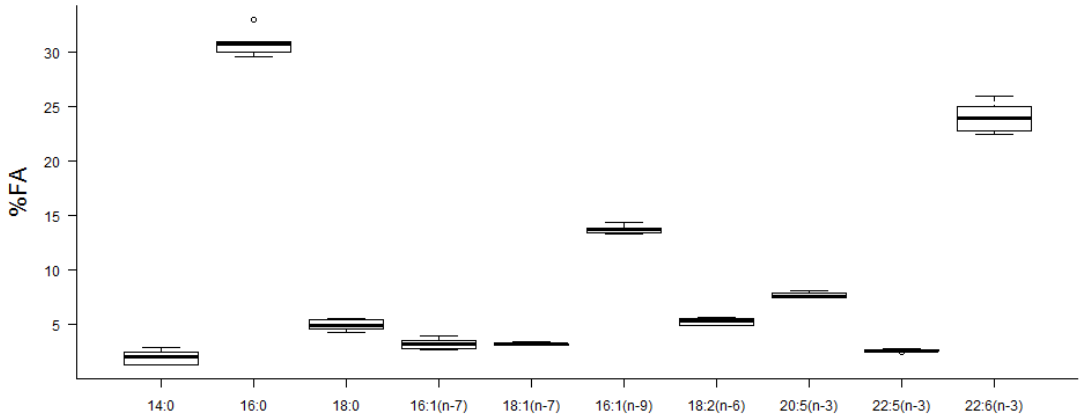

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750 **FIGURE 5**

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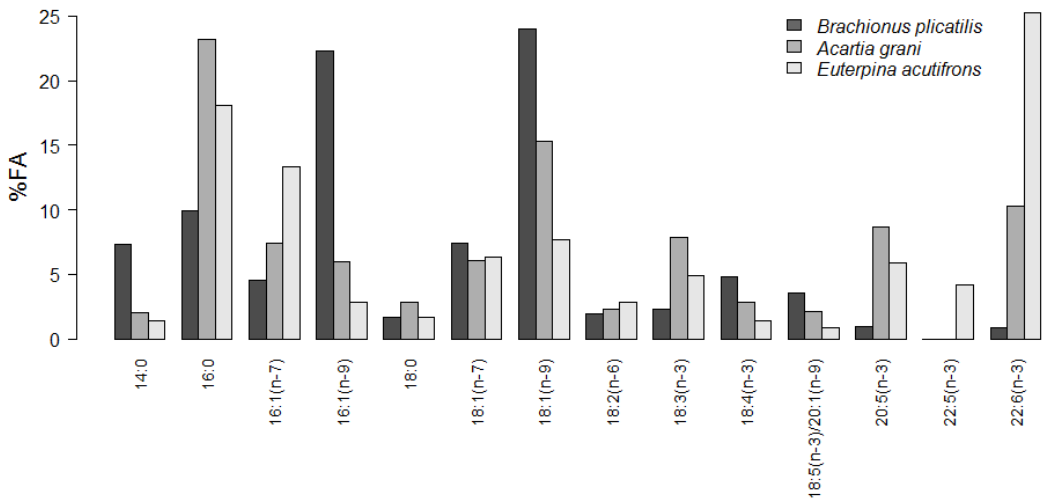

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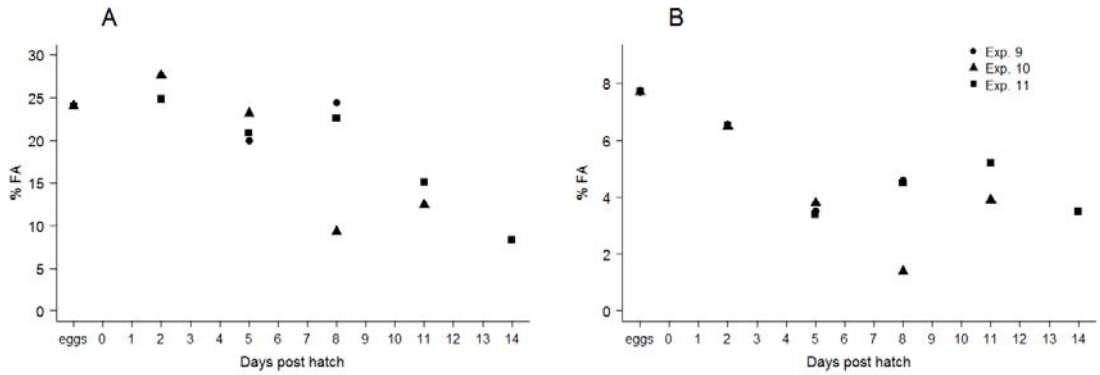
754 **FIGURE 6**



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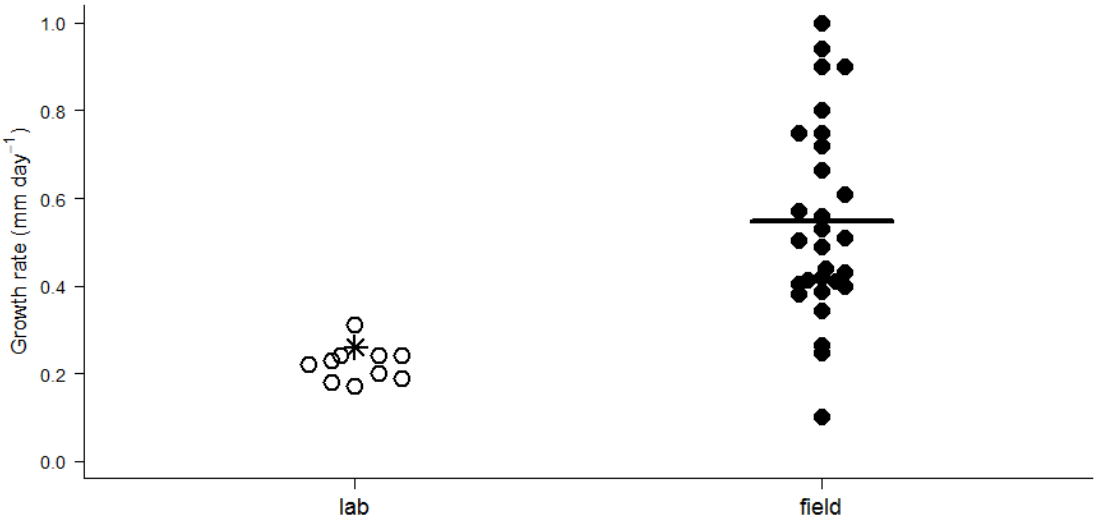
757 **FIGURE 7**



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