

1 **Co-feeding of live feed and inert diet from first-feeding affects *Artemia* lipid**
2 **digestibility and retention in Senegalese sole (*Solea senegalensis*) larvae**

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13

14 **Abstract**

15 The present study intended to evaluate the effects of early introduction of inert diet in
16 lipid digestibility and metabolism of sole, while larval feed intake, growth and survival
17 were also monitored. *Solea senegalensis* larvae were reared on a standard live feed
18 regime (ST) and co-feeding regime with inert diet (Art R). Trials using sole larvae fed
19 with *Artemia* enriched with two different lipid emulsions, containing glycerol tri [1-¹⁴C]
20 oleate (TAG) and L-3-phosphatidylcholine-1,2-di-[1-¹⁴C] oleoyl (PL), were performed
21 at 9 and 17 days after hatching (DAH) to study lipid utilization. Co-feeding did not
22 affect sole survival rates (ST 59.1 ± 15.9 %; Art R 69.56 ± 9.3 %), but was reflected in
23 significantly smaller final weight at 16 DAH (ST 0.71 ± 0.20; Art R 0.48 ± 0.14 mg).
24 Higher feed intake was observed in sole larvae fed on *Artemia* enriched with labeled PL
25 at 9 DAH but not at 17 DAH. At 17 DAH, the smaller larvae (Art R treatment) ingested

26 proportionally more *Artemia* in weight percentage, independently of enrichment. At 9
27 DAH lipid digestibility was equal among treatments and higher than 90%, while at 17
28 DAH it was higher in ST treatment (around 73 %) compared to the Art R group (around
29 66 %). Lipid retention efficiency at 9 DAH was higher in the Art R treatment, reaching
30 values of 50 %, while these values almost duplicated at 17 DAH, ranging up to 80 % in
31 both treatments without significant differences. These results show that co-feeding of
32 live feed and inert diet from first-feeding in Senegalese sole has a toll in terms of
33 growth and lipid digestibility but does not seem to compromise lipid metabolic
34 utilization.

35

36 **Keywords:** *Solea senegalensis*; Weaning; Lipid metabolism; Digestibility; Feed Intake;
37 Metamorphosis.

38

39 **1. Introduction**

40 In order to successfully achieve the objective of a significant partial replacement of live
41 feed by inert diets from first feeding, a detailed understanding of the larval digestive
42 physiology and how it may be influenced by the dietary components is indispensable
43 (e.g., Cahu and Zambonino Infante, 2001; Morais, 2005; Engrola, 2008).

44 The Senegalese sole (*Solea senegalensis*, Kaup 1858) is a flatfish found along
45 the Mediterranean and Atlantic coasts, and is a promising candidate for aquaculture in
46 Europe since the nineties due to good market prices (Howell, 1997; Dinis et al., 1999;
47 Immsland et al., 2003). Despite its high potential as an aquaculture species, only a few
48 studies looking at sole larvae rearing conditions (Esteban et al., 1995; Dinis et al., 1999)
49 and weaning strategies (Marin-Magan et al., 1995; Cañavate and Fernández-Díaz, 1999;
50 Ribeiro et al., 2002; Engrola et al., 2005, 2007, 2009a) are available. Moreover,

51 weaning success of Senegalese sole is still a critical step, with two strategies being
52 possible: sudden weaning and weaning in co-feeding with *Artemia metanauplii* (Engrola
53 et al., 2007). In spite of recent progress in sole larvae nutritional requirements and
54 understanding of larval digestive physiology, weaning results obtained so far are
55 variable and difficult to reproduce (Conceição et al., 2007b). Therefore, hatchery
56 protocols for Senegalese sole still rely on live preys during the period before the
57 metamorphosis, which occurs between 12 and 20 DAH, when they can be gradually
58 substituted by frozen *Artemia metanauplii*. Recently it has been demonstrated that
59 protein digestibility and retention are depressed by co-feeding with high levels of
60 *Artemia* replacement by inert diet, and thereby lead to lower growth (Engrola, 2008).
61 However, when a moderate level of *Artemia* replacement is used, sole are able to adapt
62 their protein metabolism and enhance protein utilization in the long term, with a growth
63 promoting effect at complete weaning (Engrola et al., 2009b).

64 Fish larvae diets, and particularly enriched *Artemia*, tend to be rich in
65 triacylglycerols (TAG) as lipid source (Morais et al., 2006), in an attempt to meet
66 essential fatty acids (EFA) requirements, namely in terms of n-3 polyunsaturated fatty
67 acids (PUFA) (Sargent et al., 1989). This can be a problem since the high levels of
68 lipids as well as the unbalances in lipid class composition found occasionally in
69 enriched live preys have been suggested to affect fatty acid (FA) digestion and
70 absorption (Salhi et al., 1995, 1997, 1999; Díaz et al., 1997; Morais et al., 2007). In the
71 marine environment, high levels of phospholipids (PL) are normally found in the total
72 lipid fraction of phytoplankton and zooplankton ingested by fish larvae (Sargent et al.,
73 1989). A beneficial effect of dietary PL supplementation in purified diets in terms of
74 survival, growth, resistance to stress, and lower occurrence of deformities has been
75 demonstrated in larval and juvenile stages of various species of fish and crustaceans

76 (Geurden et. al., 1995; Coutteau et al., 1997; Koven et al., 1998; Cahu and Zambonino
77 Infante, 2001).

78 The present work intended to evaluate the effects of co-feeding live feed with
79 inert diet from mouth opening on lipid digestive capacity and metabolism of sole larvae,
80 compared to a standard feeding regime using only live feed. To achieve this, two
81 metabolic trials using radiolabeled *Artemia* enriched with lipid emulsions containing
82 either glycerol tri [1-¹⁴C] oleate (TAG) or L-3-phosphatidylcholine-1,2-di-[1-¹⁴C]
83 oleoyl (PL) were performed with sole larvae before (9 days after hatching, DAH) and
84 during the metamorphosis climax (17 DAH). The digestibility, retention and catabolism
85 of the radiolabel incorporated in *Artemia*, as well as larval *Artemia* intake, were
86 measured. In addition, the use of different sources of radiolabeled lipid (TAG or PL)
87 enabled to verify whether these effects depend also on the molecular moiety in which
88 the FA were supplied to *Artemia*.

89

90 **2. Materials and Methods**

91 *2.1 Larval rearing*

92 Senegalese sole eggs used in the experiment were obtained from natural spawning of
93 captive breeders maintained in IPIMAR-CRIPSul, Olhão, Portugal. The larvae were
94 stocked in 100 L cylindro-conical tanks at a density of 100 larvae L⁻¹. The green water
95 technique was used in the rearing tanks with a 1:1 mixture of *Tetraselmis chuii* and
96 *Isochrysis galbana* in a recirculation system, at a temperature of 19.8 ± 0.4 °C and a
97 salinity of 37.8 ± 1.5 ‰. Oxygen saturation was 96.4 ± 9.6% and a 12/12-h light/dark
98 cycle was adopted. Water renewal was increased from 4 times/day from 0 DAH to 8
99 times/day from 13 DAH until the end of the experiment, which lasted 19 days.

100

101 2.2 Feeding regimes

102 Two different feeding regimes were randomly assigned in triplicate during the pelagic
103 phase: standard live feed (Standard, ST) and live feed co-fed with inert diet from mouth
104 opening (*Artemia* Replacement, Art R). The feeding was based on rotifers (*Brachionus*
105 *rotundiformis*) enriched with Red Pepper (BernAqua, Olen, Belgium) from 2 to 4 DAH
106 for both treatments; *Artemia* nauplii (INVE Aquaculture NV) from 4 to 9 DAH for both
107 treatments; and *Artemia* metanauplii enriched for 12 hours, at 250 nauplii mL⁻¹, with
108 0.4 g L⁻¹ in two doses (at 3 and 6h, following the manufacturer's instructions) of a 1:1
109 mixture (weight basis) of Easy DHA Selco[®] (INVE Aquaculture NV) and Micronised
110 Fishmeal[®] (Ewos, Scotland) for both treatments until the end of experiment. The
111 amount of *Artemia* supplied to the Art R treatments was gradually reduced during the
112 experiment (see Table 1). At the end of the experiment Art R sole were being offered 45
113 % frozen *Artemia* metanauplii and 55 % inert diet (*Proton* 100-200 µm; INVE
114 Aquaculture NV, Dendermonde, Belgium) in proportion (weight basis) to total daily
115 ration. Between days 13 and 16, the *Artemia* metanauplii supply to both treatments was
116 gradually changed from live to frozen *Artemia*. *Artemia* metanauplii were harvested,
117 washed in seawater, counted, and frozen at -20 °C. Fifteen minutes before feeding,
118 *Artemia* was thawed in seawater. Table 1 shows the feeding regimes in detail.

119 Table 1

120 The larvae were fed daily at 11:00 am, 14:00 pm and 17:00 pm. The first meal
121 was composed by 50 % of the daily feeding dose, and the remaining 50 % was shared
122 between the two following meals.

123

124

125

126 2.3 Sampling

127 Samples were taken for the determination of individual dry weight (DW) at: 2 DAH, at
128 mouth opening ($n=30$ per treatment), 8 DAH ($n=30$ for each replicate) and 16 DAH
129 ($n=15$ for each replicate). The larvae were stored at $-20\text{ }^{\circ}\text{C}$ and afterwards freeze-dried
130 for 48 h in a Savant SS31 (Savant Instruments Inc., Hokbrook, NY, USA). The DW of
131 the larvae was determined in a Sartorius type M5P scale (precision of 0.001mg;
132 Sartorius micro, Göttingen, Germany). Survival was determined at the end of the
133 experiment, by counting the larvae remaining in the rearing tanks.

134

135 2.4 Lipid metabolism trials

136 Two trials were performed using *Artemia* labeled with different ^{14}C -lipid sources to
137 analyze the effects of the feeding regimes on the digestive capacity and metabolism of
138 sole larvae: the first at 9 DAH, in the pelagic phase, and the second at 17 DAH, during
139 the metamorphosis climax.

140

141 2.4.1 *Artemia* [$1\text{-}^{14}\text{C}$] labeling

142 Two lipid emulsions were prepared using 0.09 g of Easy DHA Selco plus either 50 μL
143 (50 μCi) of glycerol tri [$1\text{-}^{14}\text{C}$] oleate (TAG; 3.7MBq/mL) or 100 μL (10 μCi) of L-3-
144 phosphatidylcholine-1,2-di- $[1\text{-}^{14}\text{C}]$ oleoyl (PL; 0.925MBq/mL) (Amersham Pharmacia
145 Biotech Ltd., UK). After mixing the radiolabeled lipids, the solvent in which the
146 radiolabel came dissolved was evaporated by flushing N_2 . The eppendorfs with the
147 emulsions were covered with parafilm and submitted to 5 min of ultra-sound bath at 30
148 $^{\circ}\text{C}$, vigorously shaken for 2 minutes and then stored at $-20\text{ }^{\circ}\text{C}$. The enrichment was
149 made by adding and mixing the TAG or PL emulsions to 150 mL of seawater before
150 introducing *Artemia* (200 metanauplii/mL). The incubation lasted 14 h to allow for

151 complete lipid incorporation (Morais et al., 2004b), being also a common enrichment
152 period used with several commercial products. After incubation, *Artemia* metanauplii
153 was washed thoroughly, counted and samples were taken to measure the incorporated
154 radiolabel.

155

156 2.4.2 Cold chase - set up

157 In both trials, approximately 14 h prior to the start of radiolabeled *Artemia* feeding and
158 30 min following their last meal, 10 larvae from each triplicate larval rearing tank were
159 transferred to smaller trays in the experimental radioisotope room and acclimatized.
160 Each of the four trays (two trays per treatment; one for each type of radiolabel tested)
161 contained thus 30 larvae pooled from the triplicate larval rearing tanks. An excess of
162 larvae was sampled from the rearing tanks, in relation to the requirements of the cold
163 chase trial, in case there were any mortalities during overnight acclimation or in case
164 some of the larvae would not ingest the radiolabeled *Artemia*. Each tray received
165 approximately 10,000 *Artemia* metanauplii incubated with one of the lipid radiolabels,
166 resulting in the following treatments: ST – TAG (Standard + *Artemia* incubated with
167 ^{14}C -glycerol trioleate); ST – PL (Standard + *Artemia* incubated with ^{14}C -
168 phosphatidylcholine); Art R – TAG (*Artemia* Replacement + *Artemia* incubated with
169 ^{14}C -glycerol trioleate) and Art R - PL (*Artemia* Replacement + *Artemia* incubated with
170 ^{14}C -phosphatidylcholine). Sole larvae were allowed to feed on the radiolabeled *Artemia*
171 during 30 min. This period is a trade-off between the time necessary for a complete
172 meal size and to avoid significant losses by larvae catabolism. After that time, 15 larvae
173 from each treatment presenting food in their stomachs (assessed visually) were carefully
174 transferred, one by one with an inverted Pasteur pipette, through two tanks with clean
175 seawater (to eliminate any ^{14}C -lipid that could be present in the surface of the fish), and

176 subsequently transferred to individual incubation vials. After 2 h, each larva in the
177 incubation vial was fed non labeled *Artemia* in the same concentration normally offered
178 in the rearing tanks. This feeding of radiolabeled *Artemia* followed by feeding with non
179 labeled *Artemia* characterizes a cold chase-type trial (Conceição et al., 2007a). During
180 the course of the trials, mortality was negligible - 2 dead larvae in ST PL; 1 dead larvae
181 in ArtR TG and 1 dead in ArtR PL treatments, in the first trial (9 DAH), and no
182 mortality was recorded during the second trial (16 DAH).

183

184 *2.4.3 Determination of radiolabeling*

185 The method employed allows following the metabolic fate of a tracer nutrient into
186 different compartments of individual larvae: retention in body (larvae), catabolism (CO₂
187 trap) and evacuation (incubation water) (Rønnestad et al., 2001). The metabolic
188 chambers (7.5 mL of seawater) were connected to a metabolic trap (5.0 mL of KOH 0.5
189 M), to capture the ¹⁴CO₂ eliminated by larvae. After 24 h, each larva was sampled and
190 placed in a 6 mL scintillation vial (Sarstedt, Rio de Mouro, Portugal). Hydrochloric acid
191 (HCl 0.1 M) was gradually added to the remaining water, resulting in a progressive
192 decrease of pH causing the diffusion of any remaining CO₂ in the incubation water
193 (Rønnestad et al., 2001). As a result, the radioactivity found in the water of the
194 incubation chambers corresponds to the evacuated, i.e., non absorbed, labeled *Artemia*.
195 Tissue solubilizer (Solvable, Packard Bioscience, Groningen, Netherlands) was added
196 to the vials containing the sampled larvae and the labeled *Artemia*. The vials were then
197 placed overnight in an oven to allow tissue solubilisation. After cooling, 5 mL of Ultima
198 Gold XR (Packard Bioscience) was added. To the vials containing the larva's
199 incubation water and the KOH (metabolic trap), 5 mL of Ultima Gold XR was also
200 added. The samples were counted on a Beckman LS 6000IC liquid scintillation counter

201 (Beckman Instruments Inc., Fullerton, CA, USA) and the results are presented as a
 202 percentage of disintegrations per minute (DPM) in each compartment in relation to total
 203 counts. Feed intake (FI) and feed utilization of larvae on each dietary treatment fed
 204 *Artemia* enriched with either one of the emulsions (4 combinations; $n=15$ per
 205 combination) were determined at 9 DAH and 17 DAH. Feed intake (% BDW) during
 206 the cold chase was determined as:

$$207 \quad FI = [(R_{\text{total}}/SR_{\text{Artemia}})/DW_{\text{fish}}] \times 100$$

208 as described by Conceição et al. (1998), where R_{total} is the sum of the radioactivity in
 209 the incubation seawater, in the CO₂ trap and in fish (DPM), SR_{Artemia} is the specific
 210 radioactivity in *Artemia* samples (DPM/mg *Artemia* DW), and DW_{fish} is the fish dry
 211 weight (mg).

212 Different *Artemia* utilization parameters were determined: digestibility (D, %), retention
 213 (R, %) and catabolism (C, %). These were calculated as:

$$214 \quad D = [(R_{\text{body}} + R_{\text{CO}_2 \text{ trap}})/(R_{\text{body}} + R_{\text{CO}_2 \text{ trap}} + R_{\text{water}})] \times 100;$$

$$215 \quad R = [R_{\text{body}}/(R_{\text{body}} + R_{\text{CO}_2 \text{ trap}})] \times 100;$$

$$216 \quad C = [R_{\text{CO}_2 \text{ trap}}/(R_{\text{body}} + R_{\text{CO}_2 \text{ trap}})] \times 100;$$

217 where R_{body} is the total radioactivity in fish body (DPM), $R_{\text{CO}_2 \text{ trap}}$ is the total
 218 radioactivity per CO₂ trap (DPM), and R_{water} is the total radioactivity in the incubation
 219 seawater (DPM).

220

221 *2.5 Statistical analysis*

222 The data obtained for each treatment were compared through one-way (growth and
 223 survival) or two-way (distribution of label in each compartment in metabolic trials)
 224 analysis of variance (ANOVA), using the software Statistica 6 (StatSoft Inc., Tulsa,
 225 USA). For two-way ANOVA, the combined effects of the factors “feeding regime” (ST

226 or Art R) and “lipid source” (TAG or PL) were tested. The assumption of homogeneity
227 of variance was checked using the Bartlett's test and a significance level of 0.05 was
228 employed (Zar, 1996). Data from the labeled *Artemia* feeding trial (percentage of counts
229 found in each compartment) and all other percentage data were $\arcsin(x^{1/2})$ transformed.
230 When significant differences were found in one way and two way ANOVA, the Tukey's
231 test and Newman Keuls test was performed, respectively. All data are given as mean
232 values with standard deviations (SD).

233

234 **3. Results**

235 *3.1 Larval growth and survival*

236 The initial DW of sole larvae was 42.68 ± 13.91 μg for all treatments. No significant
237 differences were observed in sole survival between the two feeding regimes (ST $59.1 \pm$
238 15.9 %; Art R 69.6 ± 9.3 %) at 16 DAH, but the final mean DW was significantly
239 higher in larvae fed live feed alone (ST treatment) at this time (Fig. 1b). However, this
240 difference in DW was still not significant at 8 DAH (Fig. 1a).

241 Figure 1

242

243 *3.2 Feed Intake*

244 The handling to transfer the larvae with the Pasteur pipette was a methodology
245 that has been previously used in other study (Engrola et al., 2009b), in which it was
246 observed that the larvae were actively feeding on *Artemia* in the incubation vials and,
247 therefore, it is believed that this factor did not affect the results, regarding the stress
248 caused to larvae.

249 *Artemia* labeling with emulsions containing either ^{14}C -TAG or ^{14}C -PL resulted in
250 average DPM values of 78.6 ± 7.5 DPM/*Artemia* and 37.9 ± 2.9 DPM/*Artemia* for TAG

251 and PL labeling, respectively, at the 9 DAH metabolic trial; and 65.8 ± 1.8 and $54.8 \pm$
252 4.9 DPM/*Artemia* for TAG and PL labeling, respectively, at 17 DAH.

253 At 9 DAH, sole fed *Artemia* enriched with PL (standard - ST - and co-feeding -Art
254 R) presented a significantly higher feed intake compared to those fed TAG-enriched
255 *Artemia* ($P < 0.001$), while no significant differences were observed between the two
256 feeding regimes. The number of *Artemia* consumed per sole larvae at 9 DAH was ST -
257 TAG 9.1 ± 2.3 ; ST - PL 23.8 ± 6.7 ; Art R - TAG 11.6 ± 4.1 ; Art R - PL 19.2 ± 6.9 .
258 Considering that there were no differences in the DW of the larva at this age, the
259 percentage of ingested *Artemia* weight in relation to the sole larva weight was
260 significantly higher in the treatments fed ^{14}C - PL labeled *Artemia* (Fig. 2a).

261 Towards the end of the experiment (17 DAH), larvae from the ST and Art R
262 treatments consumed the same amount of labeled *Artemia*, independently of the type of
263 lipid emulsion used to label them: ST - TAG 40.9 ± 16.2 ; ST - PL 41.8 ± 10.5 ; Art R -
264 TAG 43.7 ± 10.8 ; Art R - PL 42.1 ± 7.5 *Artemia*/sole larvae. Nevertheless, at 17 DAH
265 the DW of larvae reared on the standard live feed regime (ST) was higher than that on
266 Art R (Fig.1b), which means that the smallest larva (Art R) ingested proportionally
267 more *Artemia* in weight percentage ($P < 0.001$) than the sole fed live feed alone (ST
268 group) (Fig. 2b). At this time, no significant different were observed between treatments
269 fed *Artemia* enriched with either PL or TAG.

270 Figure 2

271

272 3.3 Digestibility and larval metabolism

273 There were no significant differences in *Artemia* digestibility among treatments at 9
274 DAH (Fig. 3a). At 17 DAH, however, sole from ST treatment fed with *Artemia*
275 enriched with TAG had significantly higher lipid digestibility (88.7%) ($P < 0.001$

276 “feeding regime” and $P < 0.05$ for “lipid source”). Sole from Art R treatment fed PL
277 *Artemia* had the lowest lipid digestibility, 66.0% (Fig. 3b).

278 Figure 3

279 Sole larvae at 9 DAH presented significantly higher label retention (% of label
280 absorbed) in the co-feeding treatments (Art R) ($P < 0.05$) and no statistical differences
281 were found for “lipid source” ($P > 0.07$; Fig. 4a). Lipid retention values were $48.8 \pm$
282 11.2 % in Art R – TAG; 40.4 ± 17.2 % in Art R – PL; 32.3 ± 10.6 % in ST – TAG and
283 40.0 ± 11.0 % in ST - PL. Concomitantly, the larvae submitted to the ST feeding regime
284 presented a significantly higher catabolism, when analyzed by two-way ANOVA, i.e.,
285 disregarding the lipid source utilized for *Artemia* enrichment ($67.6 \pm 10.6\%$ in ST –
286 TAG and $59.9 \pm 12.7\%$ in ST – PL; $51.1 \pm 11.2\%$ in Art R – TAG; $59.5 \pm 17.2\%$ in Art
287 R – PL). Similar results were observed when analyzing retention, catabolism and
288 evacuation expressed as DPM/ mg of sole larva DW at 9 DAH (results not shown).

289 Figure 4

290 At 17 DAH, there were no longer statistical differences in label retention or
291 catabolism (Fig. 4b) between dietary treatments and also between sole fed *Artemia*
292 enriched with either PL or TAG. The same was observed when data were expressed in
293 DPM/mg of DW (results not shown) instead of been expressed in % of DPM, even
294 when corrected for the different *Artemia* labeling (DPM/*Artemia*) in *Artemia* enriched
295 with TAG or PL.

296

297 **4. Discussion**

298 *4.1 Co-feeding inert diet from the first feeding affects larval growth but not survival*

299 The survival rates observed in the present study are in the upper range of values
300 observed in previous studies with Senegalese sole larvae (Dinis et al., 1999; Cañavate

301 and Fernández-Díaz, 1999). Furthermore, early introduction of an inert diet since mouth
302 opening (treatment Art R), did not affect the survival rate of Senegalese sole, compared
303 to the standard feeding regime until 19 DAH. Similar results were found for other
304 species (Kolkovski et al., 1997; Roselund et al., 1997), when an early co-feeding was
305 attempted. Curnow et al. (2006) co-fed Asian sea bass *Lates calcarifer* on two different
306 inert diets, and verified lower or higher survivals depending on fish size and diet type. It
307 has also been suggested that co-feeding larval sole from 1 mg of larval weight may even
308 improve survival rates at weaning, since it might enhance digestive maturation and/or
309 stimulate digestive secretion (Engrola et al., 2007), even though a higher size dispersion
310 could be observed.

311 Growth was significantly lower in the *Artemia* replacement treatment, compared
312 to sole larvae fed only on live feed until the end of the pelagic phase. However, long-
313 term effects of early co-feeding strategies in different species have been shown:
314 enhanced growth and survival after weaning in Senegalese sole (Cañavate and
315 Fernández-Díaz, 1999; Engrola et al., 2009a); increased survival rates and equivalent
316 growth in length and weight in dourado *Salminus brasilienses* (Vega-Orellana et al.,
317 2005); and improved growth and survival of tongue sole *Cynoglossus semilaevis*
318 (Chang et al., 2006). Additionally, Yúfera et al. (2003) have demonstrated that
319 Senegalese sole grew at a lower rate when fed exclusively with inert diet from 13 DAH
320 onwards, than when fed on live feed.

321 Still, even if the growth rates observed in the *Artemia* replacement treatment were
322 lower compared to the control group, they are within normal values for the Senegalese
323 sole larval rearing (Cañavate and Fernández-Díaz, 1999; Engrola et al., 2007).

324

325 *4.2 Lipid source and Artemia labeling*

326 In this study *Artemia* was labeled by including a ^{14}C -oleic acid tracer in the enrichment
327 emulsion, supplied esterified either to TAG or to PL. However, *Artemia* lipid
328 digestibility and metabolism results cannot be completely and directly related to lipid
329 class effect since it is well known that *Artemia* metanauplii cannot be considered a
330 passive carrier of FA, and both labeled TAG and PL may have been transformed into
331 other lipids by *Artemia*. In fact, an important fraction of the filtered lipids is digested,
332 assimilated into the *Artemia* body and metabolised, and not just simply accumulated in
333 the gut (Ando et al., 2004).

334 In addition to the differential metabolism of certain fatty acids, incorporated
335 fatty acids redistribute themselves among lipid classes with high unpredictability, both
336 during enrichment and particularly in starving conditions, after being added to the larval
337 rearing tanks (Watanabe et al., 1982; Léger et al., 1986, 1987; Takeuchi et al., 1992;
338 McEvoy et al., 1995, 1996; Navarro et al., 1999). In the present study, the lipid
339 composition of the radiolabeled *Artemia* at the end of the enrichment period was not
340 analyzed. Still, it is believed that the methodology used is valid to study the effect of
341 enriching *Artemia* with PL or TAG, on lipid utilization by fish larvae.

342 This is the first time that *Artemia* enriched with ^{14}C -labeled lipids is used to
343 study diet utilization in fish larvae. Previous metabolic studies have been carried out
344 using a ^{14}C -protein hydrolysate where the label was incorporated in *Artemia* protein, in
345 order to study the ontogeny of protein digestive capacity (Morais et al., 2004a) and the
346 effect of feeding regime on protein utilization (Engrola et al., 2009b).

347

348 *4.3 Artemia enrichment with phospholipids stimulates feed intake in young sole larvae*

349 At 9 DAH sole larvae fed *Artemia* enriched with radiolabeled PL presented a 2.6-fold
350 (ST) and 1.6-fold (Art R) higher feed intake when compared to the treatments fed

351 *Artemia* enriched with radiolabeled TAG. Furthermore, when the additional quantity of
352 PL supplied by the radiolabeled lipid emulsion was calculated, a very low value (0.111
353 $\times 10^{-9}$ μg of L-3-phosphatidylcholine-1,2-di-oleoyl per *Artemia*) was found. This value
354 is further reduced if it is considered that only a proportion would be found intact (i.e.,
355 non assimilated and metabolized) in the *Artemia* digestive tract at the time of larval
356 ingestion. *Artemia* enriched with labeled L-3-phosphatidylcholine-1,2-di-[1- ^{14}C] oleoyl
357 (PL) seem to have a feeding stimulation effect in sole larvae at 9 DAH. However, the
358 content of PL in the *Artemia* was not verified in the present study. Nevertheless, in
359 postlarval penaeid shrimp it was demonstrated that dietary enrichment of *Artemia* with
360 phosphatidylcholine did not enhance the *Artemia*'s PC content (Tackaert et al., 1991).
361 In gilthead seabream larvae an increased ingestion of microdiets with a higher level of
362 PC was observed (Koven et al., 1994, 1998; Izquierdo et al., 2001). The dietary PC was
363 found to be a feeding stimulant/attractant and significantly increased ingestion rates in
364 15-26 DAH gilthead seabream larvae; however it was no longer effective in 28 DAH
365 and older larvae (Koven et al., 1998). The efficacy of dietary PL thus appears to reduce
366 with age and may reflect the immature nature of the digestive system in marine fish
367 larvae which lack a fully functional digestive tract until completion of metamorphosis
368 (Munilla-Moran and Stark, 1989; Bisbal and Bengtson, 1995; Ribeiro et al., 1999; Bell
369 et al., 2003). Iritani et al. (1984) have also shown that fish larvae have limited capacity
370 for endogenous "de novo" PL biosynthesis, which may be insufficient to maintain an
371 optimal rate of lipoprotein synthesis. This is in line with Morais (2005), who suggested
372 that higher dietary requirement for PL is also probable in the earlier larval stages to
373 sustain the fast growth and organogenesis, which likely require a high rate of membrane
374 synthesis and turnover.

375 In Senegalese sole, at 17 DAH the level of ingestion of *Artemia* per sole larvae
376 was independent of the type of *Artemia* enrichment. On the other hand, the smaller
377 larvae from the Art R treatment ingested significantly more *Artemia* as percentage of
378 DW compared to sole larvae fed live feed alone. This may eventually be explained by
379 an attempt to compensate for the energy expended in metamorphosis in the *Artemia*
380 replacement larvae, together with its delay in weight gain at this stage. Similar results
381 were obtained by Engrola et al. (2009b) in similar ages.

382

383 *4.4 Early co-feeding strategy affects lipid digestive capacity of larval sole*

384 The higher *Artemia* intake when the enrichment with PL was used at 9 DAH in both ST
385 and Art R treatments was not translated into statistical differences in digestibility. At
386 this age, sole larvae seem to have a high digestive capacity to deal with lipid in live
387 preys (up to 90% digestibility), independently of their feeding regime and of the source
388 of lipid used to enrich *Artemia*. Therefore, sole early larvae seem to have better
389 digestive capacity for lipids compared to protein since protein digestibility in 8 DAH
390 sole was found to be 72.4% on a standard feeding regime and 70.4% on a co-feeding
391 regime (Engrola et al., 2009b), and 83% at 12 DAH (Morais et al., 2004a).

392 At 17 DAH a significantly lower lipid digestibility was measured in the Art R
393 treatment compared to the ST treatment and also in relation to 9 DAH. Sole larvae co-
394 fed with inert diet from mouth opening have also a reduced protein digestibility during
395 metamorphosis climax (16 DAH), compared to younger and older ages (Engrola et al.
396 2008). In fact, the metamorphosis climax is a critical developmental stage for sole
397 larvae, and early adaptation to inert diets, in particular, seems to have a toll in terms of
398 digestive efficiency. At this stage, growth, ingestion rates and oxygen consumption
399 have been shown to decrease (Parra, 1998). The higher *Artemia* lipid digestibility in

400 larvae fed the ST dietary treatment when compared with the larvae co-fed with inert diet
401 at 17 DAH, independently of the label used, may be explained by a faster development
402 of the digestive system of these larvae, related to the higher larval DW in these
403 treatments (ST). Cahu and Zambonino Infante (2001) observed that intestinal
404 maturation might be stimulated but also irreversible impaired, depending on how co-
405 feeding of live prey and inert diets is performed. In addition, Fernández-Díaz et al.
406 (2006) observed that sole exclusively fed with microencapsulated diets had altered
407 hepatic and gastrointestinal structures when compared to live feed-fed sole.

408 At 17 DAH a significantly higher digestibility was also observed in TAG-
409 labeled compared to PL-labeled *Artemia*. As already mentioned, the fatty acid and lipid
410 class composition of the radiolabeled *Artemia* was not determined and therefore the
411 higher digestibility of TAG-labeled *Artemia* has to be discussed with caution. Earlier
412 studies have showed that phospholipids have higher digestibility compared to TAG
413 (Morais et al., 2007). Still, the present study suggests that enrichment of *Artemia* with
414 PL may be less efficient to deliver oleic acid, and eventually other fatty acids, compared
415 to TAG during metamorphosis climax of sole. These findings clearly deserve further
416 study.

417

418 *4.5 Co-feeding affects lipid retention and catabolism in sole larva*

419 At 9 DAH lipid catabolism was significantly reduced and, concomitantly, body
420 retention was significantly increased, when larvae were co-fed an inert diet since first
421 feeding (Art R treatment). It has been suggested that young larvae may have the ability
422 to compensate for an eventual lower digestibility with a higher retention of absorbed
423 amino acids (Morais et al., 2004a). The same idea could be used to explain the present
424 results in relation to lipids, if it is assumed that the inert diet is less digestible than live

425 prey during the initial stages of development. In that case, larvae co-fed simultaneously
426 with live prey and inert diet might have a lower overall digestive efficiency.

427 At 17 DAH both lipid catabolism and retention presented no statistical
428 differences between treatments which, comparing with data at 9 DAH, suggests
429 metabolic changes as metamorphosis proceeds. In fact, lipid catabolism was 2-fold
430 higher (ca. 50% compared to ca. 20% of total absorbed lipid) at 9 DAH compared to 17
431 DAH. This suggests that lipid may be less important as an energy fuel during
432 metamorphosis climax. Alternatively, this may be a result of a selective pressure
433 towards an increase in body lipid as energy reserves in the stages prior to
434 metamorphosis (hence higher lipid retention), to compensate for the reduction in prey
435 consumption during this period (Youson, 1988; Parra, 1998). Then, an eventual high
436 mobilization of endogenous lipid reserves during metamorphosis climax may explain
437 the present results. Still, the present study demonstrates that lipid retention efficiency
438 remains constant independently of feeding regime and lipid source used in *Artemia*
439 enrichment during metamorphosis climax. Therefore, it seems that partial replacement
440 of *Artemia* by an inert diet does not seem to compromise larval lipid metabolism.

441

442 **5. Conclusion**

443 Co-feeding sole larvae with inert diet since mouth opening did not affect survival rate
444 but resulted in smaller size of sole larvae. Although the lipid tracer used to radiolabel
445 the *Artemia* was most likely at least partly assimilated and metabolized by the *Artemia*
446 in an unpredictable way, a fraction of the PL label remaining in the *Artemia* digestive
447 tract might have had a stimulant effect in feed intake at 9 DAH, but no longer at 17
448 DAH. In addition, while no difference was noticed at 9 DAH in lipid digestibility, the
449 ST-TAG treatment showed the highest lipid digestibility at 17 DAH, most probably as a

450 result of a more advanced maturation of the digestive system. Furthermore, at 9 DAH
451 the co-feeding regime reduced lipid catabolism and concomitantly increased lipid
452 retention in larval sole, which may be an adaptation to a feeding regime with lower
453 digestibility. However, lipid retention was high in all treatments at 17 DAH, most likely
454 as a response to morphological and physiological changes that takes place in the larval
455 body during the metamorphosis climax.

456 In short, co-feeding of live fed and inert diet from first-feeding in Senegalese
457 sole (*Solea senegalensis*) has a toll in terms of growth and lipid digestibility but does
458 not seem to compromise lipid metabolic utilization.

459

460 **6. Acknowledgements**

461 The authors wish to thank Helena Teixeira for practical assistance. Grant to Mônica Mai
462 (SWE 201887/2007-0) from “Conselho Nacional de Desenvolvimento Científico e
463 Tecnológico” - CNPq (Brasil) supported this work. This study benefited from funding
464 by Project PROMAR/SP5.P117/03 (program INTERREG III A, co-funded by FEDER,
465 European Commission).

466

467 **7. References**

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637
638

639 Figure 1 – Senegalese sole dry weight (mg) at 9 days after hatching (DAH) (1a, n = 30
640 pooled larvae) and 16 DAH (1b, n = 15). ST: Standard feeding regime; Art R: Partial
641 *Artemia* replacement by inert diet from mouth opening. Values are means \pm SD.
642 Different letters for the same age indicate statistical differences between treatments ($P <$
643 0.05, ANOVA).

644

645 Figure 2 – *Artemia* intake of sole larvae at 9 (2a) and 17 days after hatching (DAH)
646 (2b). Values are means \pm SD (2a: n=13 to ST PL; n=15 to ST TAG; n=14 to ArtR TAG;
647 n=14 to ArtR PL. 2b: n=15 for all treatments). ST – TAG (Standard feeding regime +
648 *Artemia* enriched with glycerol tri [1-¹⁴C] oleate - TAG); ST – PL (Standard feeding
649 regime + *Artemia* enriched with L-3-phosphatidylcholine-1,2-di-[1-¹⁴C] oleoyl - PL);
650 Art R – TAG (*Artemia* replacement feeding regime + *Artemia* enriched with TAG) and
651 Art R - PL (*Artemia* replacement feeding regime + *Artemia* enriched with PL). Different
652 letters at the same age indicates statistical differences ($P < 0.05$, two way ANOVA)
653 between feeding regimes (a, b) or lipid sources (x, y).

654

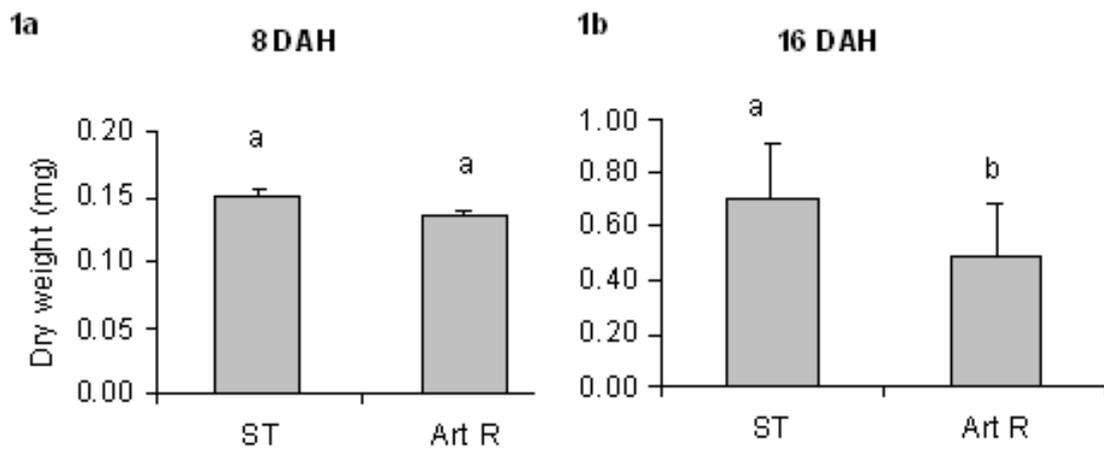
655 Figure 3 – *Artemia* lipid digestibility in sole at 9 (3a) and 17 days after hatching DAH
656 (3b). Values are means \pm SD (3a: n=13 to ST PL; n=15 to ST TAG; n=14 to ArtR TAG;
657 n=14 to ArtR PL. 3b: n=15 for all treatments). ST – TAG (Standard feeding regime +
658 *Artemia* enriched with glycerol tri [1-¹⁴C] oleate - TAG); ST – PL (Standard feeding
659 regime + *Artemia* enriched with L-3-phosphatidylcholine-1,2-di-[1-¹⁴C] oleoyl - PL);
660 Art R – TAG (*Artemia* replacement feeding regime + *Artemia* enriched with TAG) and
661 Art R - PL (*Artemia* replacement feeding regime + *Artemia* enriched with PL). Different
662 letters at the same age indicates statistical differences ($P < 0.05$, two way ANOVA)
663 between feeding regimes (a, b) or lipid sources (x, y).

664

665 Figure 4 – Lipid retention efficiency and catabolism determined in sole at 9 (4a) and 17
666 days after hatching DAH (4b). Retention (R, %) and Catabolism (C, %) were calculated
667 as: $R = [R_{\text{body}} / (R_{\text{body}} + R_{\text{CO}_2 \text{ trap}})] \times 100$; $C = [R_{\text{CO}_2 \text{ trap}} / (R_{\text{body}} + R_{\text{CO}_2 \text{ trap}})] \times 100$;
668 respectively, where R_{body} is the total radioactivity in fish body (DPM) and $R_{\text{CO}_2 \text{ trap}}$ is the
669 total radioactivity per CO_2 trap (DPM).

670 Values are means \pm SD (4a: n=13 to ST PL; n=15 to ST TAG; n=14 to ArtR TAG;
671 n=14 to ArtR PL. 4b: n=15 for all treatments). ST – TAG (Standard feeding regime +
672 *Artemia* enriched with glycerol tri [$1\text{-}^{14}\text{C}$] oleate - TAG); ST – PL (Standard feeding
673 regime + *Artemia* enriched with L-3-phosphatidylcholine-1,2-di- $[1\text{-}^{14}\text{C}]$ oleoyl - PL);
674 Art R – TAG (*Artemia* replacement feeding regime + *Artemia* enriched with TAG) and
675 Art R - PL (*Artemia* replacement feeding regime + *Artemia* enriched with PL). Different
676 letters at the same age indicate statistical differences ($P < 0.05$, two way ANOVA)
677 between feeding regimes (a, b).

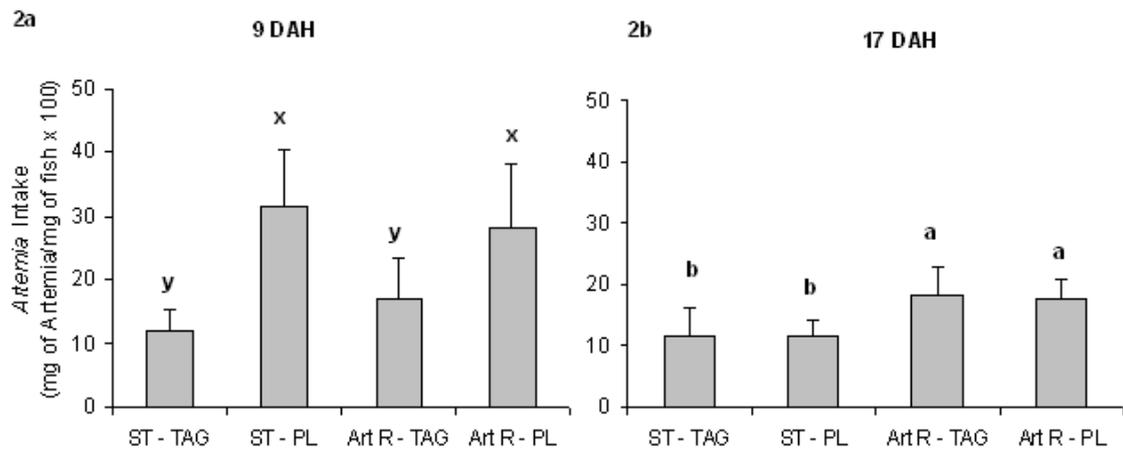
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680 Fig 1

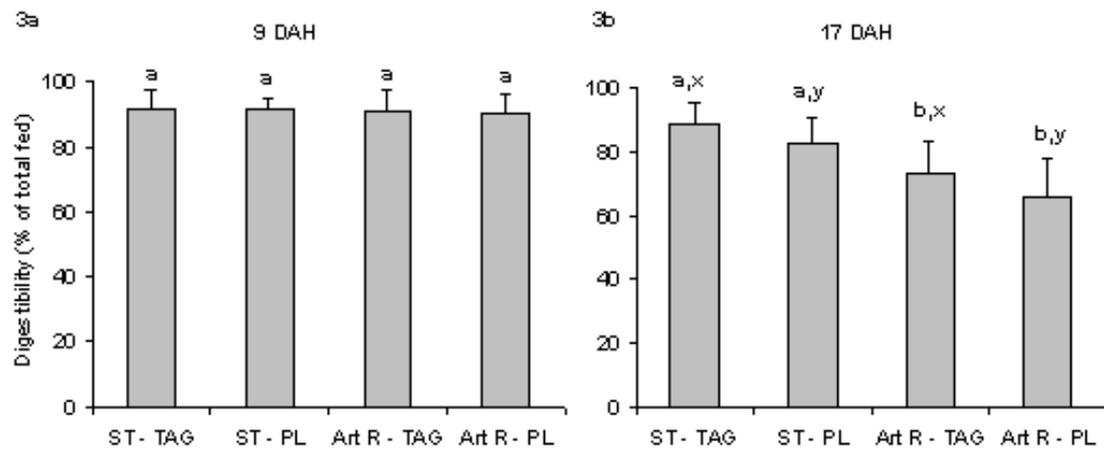
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683 Fig 2

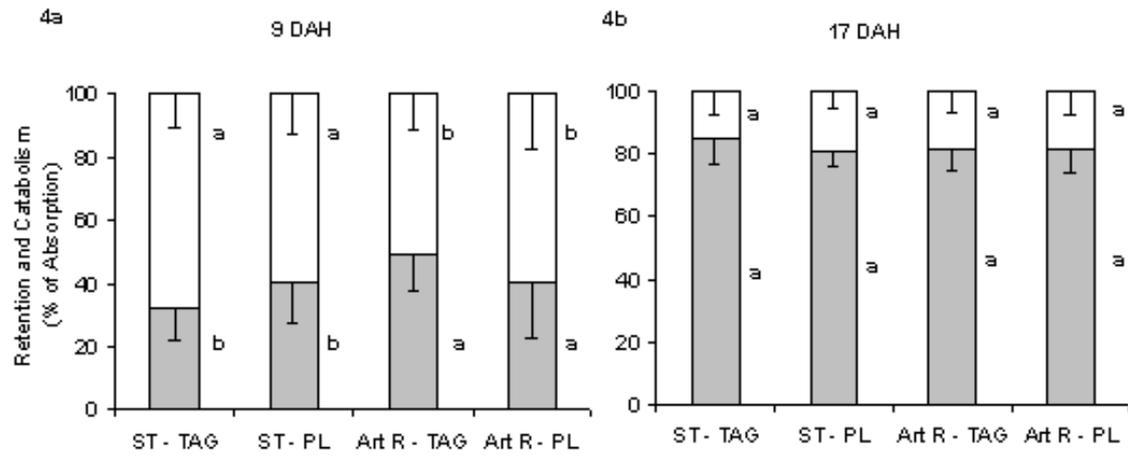
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686 Fig 3

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689 Fig 4