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Effects of light intensity on growth, feeding activity and development in common sole (*Solea solea* L.) larvae in relation to sensory organ ontogeny

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1 **Effects of light intensity on growth, feeding activity and development in common**
2 **sole (*Solea solea* L.) larvae in relation to sensory organ ontogeny**

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16 Running title: Light intensity in common sole larvae

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18

19 *Keywords:* *Solea solea*; Fish larvae; Light; Sensory organs; Growth; Histology

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24

25 **Abstract**

26

27 The effects of four light intensities (1000 lx, 500 lx, 50 lx, 3 lx) on growth, survival and
28 feeding activity in common sole (*Solea solea* L.) larvae were studied from 4 till 51 days
29 post hatching (dph). During the pelagic larval stage (4-12 dph), larvae reared at 3 lx
30 showed a lower growth. From 19 onwards, the larvae reared under 3 lx displayed a
31 significant ($P \leq 0.05$) higher SGR than the other treatments and a higher final weight
32 compared to 1000 lx and 500 lx. Survival rate was higher under intermediate light
33 intensities (500 and 50 lx). Larvae reared at 3 lx displayed a significant delay in the
34 degree of metamorphosis compared to the other treatments, while at 33 dph
35 metamorphosis was completed under all treatments. Histological examination revealed
36 the importance of vision and light in the first feeding of this species, while after
37 metamorphosis, the full development of other sensory organs indicated that feeding
38 activity is also mediated by chemosensory perception. Results indicate that high light
39 intensity seems to be more suitable during the pelagic larvae, while the opposite would
40 ensure better growth from the onset of metamorphosis to the benthic phase.

41

42 **Introduction**

43

44 Abiotic factors, such as water temperature, salinity, oxygen and light, are known to
45 influence the growth, survival, behavior and development of fish (Howell & Baynes
46 2004). One of the key environmental parameters is light, which is considered as a
47 complex set of characteristics such as intensity, photoperiod and spectrum (Villamizar,
48 Blanco-Vives, Migaud, Davie, Carboni & Sánchez-Vázquez 2011). Light has been
49 shown to influence fish throughout various stages of their life, from embryonic
50 development to the sexual maturation of adults (Boeuf & Le Bail 1999; García-López,
51 Pascual, Sarasquete & Martínez-Rodríguez 2006; Stuart 2013; Villamizar *et al.* 2011).
52 Among light characteristics, light intensity has been defined as the amount of
53 illumination at the water surface, per unit area (Cobcroft, Pankhurst, Hart & Battaglene
54 2001; Stuart & Drawbridge 2011). Knowledge about the sensitivities to this parameter
55 in fish larvae is still scarce, though it is an extremely important environmental factor in
56 larval rearing and known to significantly affect growth, development and survival
57 (Stuart 2013; Villamizar *et al.* 2011). Light intensity can be also responsible for the
58 larvae initiating feeding and can affect the ability to forage, since many marine fish
59 larvae are visual feeders and require a minimum amount of light to feed (Hubbs &
60 Blaxter 1986; Fielder, Bardsley, Allan & Pankhurst 2002; Monk, Puvanendran &
61 Brown 2006; Puvanendran & Brown 2002). The feeding responses of larvae in different
62 light environments change with the developing visual capabilities of larvae and have
63 been correlated with larval distribution in the wild (Job & Bellwood 2000) and used to
64 determine appropriate light conditions for larval culture (Pankhurst & Hilder 1998).

65 On the other hand, high light intensity may also negatively modify larval behavior
66 (Stuart 2013) and can be stressful or even lethal for larvae (Boeuf & Le Bail 1999).
67 Light requirements in terms of intensity in which fish can thrive are species-specific and
68 the optimal values for larval development, growth and survival may also differ during
69 larval ontogeny (Boeuf & Le Bail 1999; Fielder *et al.* 2002; Stuart 2013).

70 Recently, some improvements in the study of common sole larvae (*Solea solea* L.) have
71 been achieved by focusing on larval feeding, nutrition and physiology (Bonaldo, Parma,
72 Badiani, Serratore & Gatta 2011; Ferraresso, Bonaldo, Parma, Cinotti, Massi,
73 Bargelloni & Gatta 2013; Lund, Steinfeldt & Hansen 2007; Lund, Steinfeldt, Banta &
74 Hansen 2008; Parma, Bonaldo, Massi, Yufera, Martínez-Rodríguez & Gatta 2013).
75 However, abiotic factors such as light intensity at the larval stage are poorly
76 investigated in this species and, to our knowledge, the study carried out by Lund,
77 Steinfeldt & Hansen (2010) is the only reference available on this topic.

78 We thus investigated the effects of light intensity on growth, feeding activity and
79 development in common sole larvae with the aim of providing useful information for
80 hatcheries. To this end, common sole larvae were exposed to four different light
81 intensity regimes, from 3 to 1000 lux, following previous studies on other marine
82 flatfish (Cañavate *et al.* 2007; Henne & Watanabe 2003; Huse 1994). The trial lasted 48
83 days, covering the entire sole larval cycle, from before to after metamorphosis. To gain
84 further insight into larval development, we also sought to provide a comprehensive
85 description of the major organs, with special regard to the ontogeny of sensory organs,
86 based on histological examination.

87

88 **Materials and methods**

89

90 *Experimental design and rearing system*

91

92 Four treatments were tested in triplicate in order to investigate different light
93 intensity regimes (LRs): light intensity of 1000 lx (LR-1000), light intensity of 500 lx
94 (LR-500), light intensity of 50 lx (LR-50) and light intensity of 3 lx (LR-3) at the water
95 surface.

96 The experimental facility consisted of twelve 280-liter square grey (RAL 7038) flat
97 bottom (0.64 m²) tanks supplied with natural seawater and connected to a closed
98 recirculating system (overall water volume: 4000 L). The rearing system consisted of a
99 mechanical sand filter (0.4 m³ of silica sand, 0.4-0.8 mm. PTK 1200, Astral Pool,
100 Servaqua S.A. Barsareny, Spain), ultraviolet lights (PE 25mJ/cm²: 16m³ h⁻¹, Blaufish,
101 Barcelona, Spain) and a biofilter (PTK 1200, Astral Pool, Servaqua S.A. Barsareny,
102 Spain). Each tank was provided with artificial white light supplied by a dimmable lamp
103 (Disano Hydro 951, Disano Illuminazione S.p.A, Rozzano, Italy; Philips master TL-
104 D18 W/840, Philips S.p.A, Monza, Italy) with two bulbs, one lamp for each tank placed
105 at 60 ± 2 cm from the water surface.

106 In order to obtain a low light intensity for treatment LR-3, the tanks were separated
107 from the other tanks by black plastic sheeting, which were fixed from the floor to the
108 ceiling, to eliminate extraneous light.

109 At the beginning of the trial, light intensity was regulated (Delta Ohm lightmeter
110 HD2302.0; Probe LP 471 PHOT; Delta Ohm, Padua, Italy) at the water surface
111 according to the LRs and values were monitored daily, in the morning before feeding to
112 avoid large variations. Averages recorded were 1052.7 ± 96.6 lx under LR-1000, 512.0

113 ± 21.4 lx under LR-500, 53.0 ± 8.9 lx under LR-50 and 2.8 ± 0.6 lx under LR-3. The
114 irradiance (rad, W/m^2) at the water surface of each tank was also measured at the same
115 time (Delta Ohm lightmeter HD2302.0; Probe LP 471 RAD; Delta Ohm, Padua, Italy).
116 Averages recorded during the trial were 2.705 ± 0.203 W/m^2 , 1.323 ± 0.145 W/m^2 ,
117 0.146 ± 0.036 W/m^2 and 0.007 ± 0.002 W/m^2 in the LR-1000, LR-500, LR-50 and LR-3
118 treatments respectively. The trial lasted 48 days, from 4 days post hatching (dph) to 51
119 dph.

120

121 *Larvae origin and feeding*

122

123 Common sole larvae were obtained from a captive broodstock maintained at the
124 Laboratory of Aquaculture, Department of Veterinary Medical Sciences, Cesenatico,
125 Italy. One batch of fertilized floating eggs was incubated in an 80 L cylinder-conical
126 tank until 4 dph, under a light intensity of 160 lx. At this time, 2050 larvae tank⁻¹ were
127 randomly distributed to the experimental rearing tanks using volumetric allocation
128 (Bonaldo *et al.* 2011). The feeding protocol for all LRs consisted in the administration
129 of live feed (*Artemia* nauplii from 4 to 10 dph, subsequently enriched metanauplii) until
130 18 dph, followed by weaning onto a commercial microdiet (MD) (AgloNorse K/S
131 Tromsø Fiskeindustri A/S & Co., Tromsø, Norway) (Parma *et al.* 2013). *Artemia*
132 nauplii and metanauplii were manually administered twice a day (10.00 am and 4.00
133 pm). Live feed was added so as to reach an overall amount of 10 individuals $\text{mL}^{-1} \text{day}^{-1}$,
134 and 3 days before the end of weaning (18 dph) it was gradually decreased to 2
135 individuals $\text{mL}^{-1} \text{day}^{-1}$. The diet was supplied from 10 to 33 dph at a commercial size
136 of 200-300 μm and subsequently at a commercial size of 300-500 μm and 400-600 μm .

137 Dry feed was supplied by belt feeders for 12 h day⁻¹. Larvae were fed in excess to
138 guarantee a high availability of MD particles in the water and avoid an excess of
139 uneaten feed on the tank bottom. The feeding rate was adjusted after a daily visual
140 inspection of the tank bottoms, which took place in the early morning (8.30 am) before
141 feed was supplied. The daily feed ratio was decreased in the case of excessive leftover
142 feed (Parma *et al.* 2013). Feed administration ranged from 4 to 8 g tank⁻¹ day⁻¹. *Artemia*
143 cysts (Great Salt Lakes, Catvis BV, s-Hertogenbosch, the Netherlands) were incubated
144 and hatched in seawater (salinity 25 g l⁻¹) at 28 °C over an 18 h period. *Artemia*
145 metanauplii were harvested and enriched for 24 h using Algamac-3050 (Aqua fauna,
146 Bio-Marine Inc., Hawthorne, CA, USA).

147

148 *Water quality – Environmental parameters*

149

150 During the trial, the water temperature was kept constant at 18.0 ± 1.0 °C and the
151 photoperiod was maintained at 16 h light: 8 h dark. The oxygen level was kept constant
152 (7.5 ± 1.0 ppm) using a liquid oxygen system connected to a software program (B&G
153 Sinergia snc, Chioggia, Italy); furthermore strong aeration (200 l min⁻¹) was applied in
154 the stock tank to remove CO₂. Overall daily water renewal was approximately 20 %
155 day⁻¹ as a consequence of mechanical filter backwashing and tank flushing. At the
156 beginning of the trial, the water exchange rate of the tanks was 1 L min⁻¹, and it was
157 increased to 2 L min⁻¹ following larval development. Ammonia (total ammonia nitrogen
158 ≤ 0.1 ppm), nitrite (≤ 0.2 ppm), nitrate (≤ 50 ppm) and salinity (37 g L⁻¹) were
159 monitored daily by spectrophotometry in the afternoon (5.00 pm) (Spectroquant Nova

160 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on a
161 daily basis to keep the pH constant at 7.8 - 8.0.

162

163 *Data collection, growth, survival and metamorphosis*

164

165 At the beginning of the trial, 3 pools of 50 larvae each were randomly collected from
166 the initial stock for wet weight (WW) determination.

167 At 12, 19, 33 and 51 dph, 50 larvae tank⁻¹ were collected to determine WW. WW
168 was measured with a microbalance (Scaltec SBC31, Scaltec Instruments GmbH,
169 Göttingen, Germany) after rinsing the larvae with deionized water on a 400 µm screen
170 and blotting excess moisture away from behind the screen using a lint free paper towel
171 (Bonaldo *et al.* 2011). The growth rate was calculated as the specific growth rate (SGR,
172 % day⁻¹), according to the following equation:

173
$$\text{SGR} = \frac{(\ln \text{ final weight}) - (\ln \text{ initial weight})}{\text{Number of Days}} \times 100$$

174 Twenty larvae per tank were examined using a stereomicroscope (Nikon SMZ 800,
175 Nikon Instruments Inc. Melville, NY, USA) to determine the degree of metamorphosis,
176 evaluated by scoring the position of the migrating left eye. Degrees of metamorphosis
177 were divided into 5 phases (S): 0) symmetrical left and right eye position; 1) an
178 asymmetrical position of the left eye and right eye, the left eye starts to migrate; 2) the
179 migrating eye reaches at maximum the midline of the dorsal surface; 3) the migrating
180 eye can be seen from the right ocular side or migrates within the dorsal side; 4) eye
181 translocation is completed and the orbital arch is visible (Bonaldo *et al.* 2011).

182 At the end of the trial, the remaining larvae were removed from each tank and counted
183 to determine the survival rate (SR) as a percentage of the initial number of larvae.

184 All experimental procedures were evaluated and approved by the Ethical-Scientific
185 Committee for Animal Experimentation of the University of Bologna, in accordance
186 with European directive 2010/63/EU on the protection of animals used for scientific
187 purposes.

188

189 *Gut fullness*

190

191 At 6, 8, 10 and 13 dph, 10 larvae tank⁻¹ were collected to determine gut fullness.
192 Larvae were sampled in the morning (1 h after the meal), anesthetized in
193 phenoxyethanol and immediately observed under a microscope (Nikon SMZ 800, Nikon
194 Instruments Inc. Melville, NY, USA). At 6, 8 and 10 dph, gut fullness was determined
195 by counting the number of *Artemia* ingested, according to Downing & Litvak (2001)
196 and Blanco-Vives, Aliaga-Guerrero, Cañavate, García-Mateos, Martín-Robles, Herrera-
197 Pérez, Muñoz-Cueto & Sánchez-Vázquez (2012). Whole prey or partially digested
198 *Artemia* were clearly visible within the larval gut. At 13 dph, due to the impossibility of
199 clearly distinguishing and counting the *Artemia*, fullness of gut was instead visually
200 estimated through the observation of microscope images and classified into four
201 categories (nominally: empty, medium, full and very full) (Mc Lauren & Avendano
202 1995; Puvanendran & Brown 2002).

203

204 *Histology*

205

206 At the beginning of the trial, a pool of 9 larvae, and subsequently 5 larvae per tank per
207 sampling day (8, 14, 18, 25, 34 and 51 dph), were collected. Samples were fixed in 10%
208 buffered formalin and paraffin embedded. Sections were cut at 3 μm and stained with
209 hematoxylin-eosin. The sections were photographed with a camera (Nikon Digital Sight
210 SD-MS, Tokyo, Japan) connected to an optical microscope (Nikon Eclipse 80i, Tokyo,
211 Japan). A microscopic evaluation was performed, focusing on the major developing
212 organs, with special regard to sensory organs (eyes, olfactory sensory cells, otic
213 vesicles, neuromasts, taste buds and barbels). As histological studies for common sole
214 larval development are lacking, we adopted the approach used in the study by Padrós,
215 Villalta, Gisbert & Estévez (2011) on Senegal sole larvae.

216

217 *Statistical analyses*

218

219 The results in terms of WW, SGR, SR and gut fullness at 6, 8, 10 dph were analyzed
220 using one-way ANOVA, followed by Tukey's Multiple Comparison Test. Gut fullness
221 at 13 dph and metamorphosis data was analyzed using Pearson's χ^2 test with Yates'
222 continuity correction. All statistical analyses were performed using the statistical
223 package R version 2.15.3 for Windows (Revolution analytics, Palo Alto, CA, USA) and
224 GraphPad Prism 5.0 for Windows (Graph Pad Software, San Diego, CA, USA). The
225 differences between treatments were considered significant at $P \leq 0.05$.

226

227 **Results**

228

229 At the end of the trial, the SR was significantly higher in the LR-50 treatment
230 compared to the LR-1000 and LR-3 treatments; the LR-1000 treatment showed a lower
231 SR than both the LR-500 and LR-50 treatments (Table 1). The SGR data are presented
232 in Table 1. The SGR of the larvae of the LR-3 treatments was significantly lower than
233 in all the other treatments until 12 dph. From 12 to 19 dph, the larvae of the LR-3
234 treatment had a higher SGR than the LR-50 and LR-500 treatment. From 19 to 51 dph,
235 the LR-3 treatment had a significantly higher SGR than under all the other LRs. The
236 SGR of the LR-50, LR-500 and LR-1000 treatments were not significantly different
237 during the trial.

238 Table 1.

239 The larvae of the LR-3 treatment showed a significantly lower WW than those of all
240 the other treatments at 12 dph and of those of the LR-1000 treatment at 19 dph,
241 respectively (Fig. 1). At 33 dph, there were no significant differences among treatments,
242 whereas at the end of the trial (51 dph), the larvae of the LR-3 treatment showed a
243 higher WW in comparison with those of the LR-1000 and LR-500 treatments.

244 Figure 1.

245 The gut fullness data are presented in Fig. 2. At 6 dph, the LR-3 treatment showed a
246 lower number of ingested prey compared to the LR-500 treatment. At 8 dph, gut
247 fullness of the LR-3 treatment was lower than that of LR-50 and LR-1000 treatments,
248 whereas at 10 dph, the same treatment showed a lower value compared to the LR-500
249 and LR-1000 treatments (Fig 2, a). At 13 dph, the LR-3 treatment showed a significant
250 lower fullness of gut compared to all the other treatments (Fig 2, b).

251 Figure 2 a, b.

252 For most of the specimens observed (S1, 93-100%), metamorphosis started at 12 dph
253 in the LR-1000, LR-500 and LR-50 treatments (Fig. 3). In the LR-3 treatment, the
254 larvae displayed a significant delay in the degree of metamorphosis development at 12
255 dph compared to the larvae in the other treatments (S1, 68%). At 20 dph, the larvae in
256 the LR-1000 treatment showed a significant advance in the degree of metamorphosis in
257 comparison with the other treatments and the LR-500 treatment showed a higher degree
258 compared to the LR-3 treatment. From 24 dph onward, no significant differences were
259 observed among the treatments and at 33 dph metamorphosis was completed (S4) under
260 all the LRs.

261 Figure 3.

262 No histopathological changes were observed in any individual larvae. At 5-8 dph,
263 eyes were fully pigmented and the retina showed, in the outer nuclear layer (ONL), the
264 presence of retinal stem cells, 15 μm in diameter, with a centrally located,
265 hyperchromatic round nucleus at the base of the photoreceptor layer (PRL) (Fig. 4, a).
266 Olfactory bulbs and otic vesicles appeared; olfactory sensory cells were arranged in two
267 small olfactory placodes. Neuromasts were seen in the epidermal layer covering the
268 cephalic region; taste buds appeared in the ventral pharyngeal region and along the
269 alimentary canal. The yolk sac was fully reabsorbed and gut content was visible.
270 Several mucous cells appeared in the esophageal epithelium and the intestine was
271 divided in two portions. The liver and exocrine pancreas were well developed and
272 hepatocytes contained a large amount of lipid/glycogen. The swim bladder was visible
273 above the gut. Large melanophores (larval type) were arranged in groups in the cephalic
274 region and trunk. Several epidermal mucous cells appeared in the integument.

275 At 14-18 dph, in some specimens both eyes were visible on the right side. A few retinal
276 stem cells were still present within the ONL. Olfactory cavities and nostrils were
277 present. Otoliths were clearly evident within the otic vesicles. Neuromasts (Fig. 4, b)
278 and taste buds extended along the alimentary canal, reaching the anatomic region
279 corresponding to the stomach. Barbels appeared around the mouth. The kidney appeared
280 well differentiated with evident collecting ducts. The heart and spleen were well evident
281 and formed. Numerous thyroid follicles filled with colloid were evident. Small
282 melanophores of an adult type appeared together with large melanophores of a larval
283 type.

284 At 25 dph, the metamorphosis of the larvae observed had been completed. The olfactory
285 bulbs increased in size, the otic vesicles became larger and otoliths were clearly
286 detectable inside them. Neuromasts were visible also in the ventral aspect of the body.
287 Taste buds were widely distributed in the buccal cavity and esophagus (Fig. 4, c). The
288 brain was fully developed (telencephalon, hypothalamus and myelencephalon,
289 cerebellum). Pigmentation was similar to that of adults.

290 After 34 dph and up to 51 dph, all subjects were closely similar to adults; the sensory
291 organs were fully developed and further changes were only related to increases in size
292 (Fig. 4, d).

293 Figure 4.

294

295 **Discussion**

296

297 Successful mass production of high-quality juveniles is dependent on successful larval
298 rearing. The determination of optimal environmental conditions for fish larvae,

299 including light, is important to maximize their growth and survival (Stuart 2013). A
300 better understanding of light requirements during larval rearing may aid in standardizing
301 the optimal culture conditions. The literature shows that light requirements at larval
302 stage are species specific with a wide range of optimal values found among species,
303 extending from a few lux for Atlantic cod (*Gadus morhua* L.) to thousands of lux for
304 California yellowtail (*Seriola lalandi*) (Valenciennes) (Table 2).

305 Table 2.

306 In the present study, common sole showed a different response in relation to light
307 intensity and larval stage. During the first period of the trial (from 4 to 12 dph), which
308 corresponds to the pelagic larval stage, a low light intensity (3 lx) negatively affected
309 WW and SGR and the amount of prey ingested during the pelagic stage seemed to be
310 negatively affected by the lowest light intensity. Hence, the lower growth recorded at 12
311 dph for the larvae reared under 3 lx compared to the other treatments may be
312 attributable to their reduced capability of detecting and capturing live feed, which is
313 consistent with general finding in the pelagic stage of marine fish larvae (Monk *et. al*
314 2006). According to the histology observation, sole larvae observed at 5-8 dph showed
315 undeveloped sensory organs with the exception of eyes, which were fully pigmented
316 and therefore functional enough for feeding activity (Roo, Socorro, Izquierdo,
317 Caballero, Hernández-Cruz, Fernández & Fernández-Palacios 1999; Cobcroft &
318 Pankhurst 2003; Bejarano-Escobar, Blasco, DeGrip, Oyola-Velasco, Martín-Partido &
319 Francisco-Morcillo 2010; Ortiz-Delgado, Iglesias, Sánchez, Cal, Lago, Otero &
320 Sarasquete 2012). However, the presence in the ONL of retinal stem cells, which are
321 recognized as the rod precursors (Otteson & Hitchcock 2003), suggest that vision at this

322 developmental stage is mainly mediated by cones, which limit vision at relatively high
323 light intensity conditions in superficial waters during the day (Hubbs & Blaxter 1986).
324 Different studies have shown that the majority of marine fish larvae are mainly diurnal
325 visual feeders (Boeuf & Le Bail 1999; Peña, Dumas, Saldivar-Lucio, García, Trasviña
326 & Hernández-Ceballos 2004; Puvanendran & Brown 2002) and a positive correlation
327 between high light intensity and increased growth (Henne & Watanabe 2003; Huse
328 1994; Monk *et al.* 2006; Puvanendran & Brown 2002; Vallés & Estévez 2013) has been
329 found. Visual feeding behavior has also been described in Senegal sole larvae, where
330 the capture of live prey during the early pelagic stage depended on light (Cañavate,
331 Zerolo & Fernández-Díaz 2006). On the other hand, our results suggest that an
332 increment of light intensity from 50 to 1000 lx does not bring about any improvement in
333 prey ingestion, WW or SGR at 12 dph. This last finding was also observed in Senegal
334 sole larvae (Cañavate *et al.* 2007), where different light intensities, in the range of 200
335 to 2000 lx, had no effect on growth from 3 to 20 dph.

336 From 12 dph onwards, following the onset of metamorphosis, an inverse growth trend
337 began to emerge, which became more evident in the last part of the trial (settlement and
338 benthonic phase). In fact, between 12 and 19 dph, the larvae reared under 3 lx displayed
339 a higher SGR than those under 50 and 500 lx, while between 19 and 51 dph their
340 growth rate was above that under all the other LRs. As a consequence, the growth in
341 terms of WW was similar among treatments at 33 dph, while at 51 dph the larvae reared
342 under 3 lx displayed a higher WW compared to those under 500 and 1000 lx.
343 Metamorphosis showed a different pattern of development. At 12 and 20 dph, the larvae
344 reared at the lowest light intensity displayed a delay in the degree of metamorphosis
345 while at 24 and 33 dph metamorphosis had been completed in all the LR treatments. A

346 dietary energy limitation during the larval stage has been pointed out as a relevant factor
347 that may prevent fish from reaching the energy reserves required for the onset of
348 metamorphosis (Parra & Yúfera 2001; Pinto 2013). Some authors have reported that
349 common sole and Senegal sole larvae continue feeding during metamorphosis (Amara,
350 Lagardere & Desaunay 1993; Fernández-Díaz, Yúfera, Cañavate, Moyano & Díaz
351 2001; Geffen, Van der Veer & Nash 2007), suggesting that the matter and energy
352 required for transformation do not originate exclusively from body reserves.
353 Furthermore, our own findings suggest that the degree of metamorphosis was mainly
354 related to the weight of the larvae and the influence of light intensity on eye migration
355 seems negligible.

356 During this period, the most pronounced physiological and behavioral changes of larvae
357 are related to the shift from pelagic to benthic life (Fernández-Díaz *et al.* 2001;
358 Yamashita, Tanaka & Miller 2001). In the wild, these habitats are characterized by
359 different lighting conditions, since light intensity decreases exponentially with depth in
360 the water column (Thistle 2003). At the end of metamorphosis, members of this species
361 spend most of their life on the sea bottom, generally up to 30 and 100 meters of depth
362 for juveniles and adults, respectively (Salen-Picard, Darnaude, Arlhac & Harmelin
363 Vivien 2002; Grati, Scarcella, Polidori, Domenichetti, Bolognini, Gramolini, Vasapollo,
364 Giovanardi, Raicevich, Celić, Vrgoč, Isajlovic, Jenič, Marčeta & Fabi 2013).

365 Furthermore, sole species such as Senegal sole switch from a diurnal behavioral pattern
366 to nocturnalism during larval metamorphosis (Blanco-Vives *et al.* 2012) and their
367 ability to feed in darkness seems to improve with age (Cañavate *et al.* 2006).

368 Histology evidences that after metamorphosis feeding activity is also mediated by
369 chemosensory perception. In fact, observation revealed a wide distribution of

370 neuromasts and taste buds, besides the appearance of barbels around the mouth with
371 growth. Moreover, the development of a sophisticated olfactory epithelium, very rich in
372 microvillar and ciliated cells, supports the enhanced olfactory capability required during
373 the benthonic phase (Appelbaum, Adron, George, Mackie & Pirie 1983). Neuromasts
374 are sensory receptors that respond to mechanical stimuli and are probably involved, in
375 low light conditions, in the capture of *Artemia*, which produce vibrations (Mukai & Lim
376 2012). Taste buds and the olfactory epithelium respond to chemical stimuli such as free
377 amino acids released from live prey and MD (Mukai, Tuzan, Lim & Yahaya 2010),
378 while barbels are mainly necessary in benthic species to recognize the sea bottom and
379 search for prey items (Lombarte & Aguirre, 1997).

380 SR showed a different trend compared to WW and SGR. In fact, at the end of the trial,
381 SR was higher for larvae reared under an intermediate light intensity (500 and 50 lx).
382 Boeuf & Le Bail (1999) reported that in larvae optimal light for growth is often not the
383 same as for survival, because too much light can be stressful or even lethal. Similar
384 results were found in Southern flounder larvae, where survival at 11 and 15 dph was
385 generally higher under a mid-range intensity compared to the extreme values (Henne &
386 Watanabe 2003). The authors suggested that while the minimum illumination threshold
387 theory explains the poor growth and survival of marine fish larvae under low light
388 intensities, there appears to be a maximum illumination level above which larval growth
389 and/or survival are adversely affected in some species.

390 In summary, common sole larvae require specific light intensities to maximize feeding
391 activity, growth and survival. Based on the results of the present trial, we suggest
392 different light regimes during larval ontogeny: a high light intensity seems to be the
393 most suitable for first-feeding larvae, while a low light intensity would ensure better

394 growth after larval settlement. Thus, the light intensity regime can be fixed between 50
395 and 500 lx during the pelagic stage (4-12 dph) and reduced to 3-50 lx from the onset of
396 metamorphosis to the benthic phase. Further investigations are needed to determine the
397 effects of environmental factors on the early life stages and larval feeding behavior of
398 this species.

399

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404 ciclo produttivo della sogliola comune *Solea solea*: analisi e risoluzione dei punti critici
405 e valutazione economica” (*Feasibility study on the entire production cycle of the
406 common sole Solea solea: analysis and resolution of critical aspects and economic
407 assessment*), invito DG Pamac Segreteria Prot. Uscita 0042305, 25/11/2001.

408

409 **References**

410

411 Amara R., Lagardere F. & Desaunay Y. (1993) Seasonal distribution and duration of
412 the planktonic stage of Dover sole, *Solea solea*, larvae in the Bay of Biscay: an
413 hypothesis. *Journal of Fish Biology* **43** (Suppl. A), 17-30.

414 Appelbaum S., Adron J.W., George S.G., Mackie A.M. & Pirie B.J.S. (1983) On the
415 development of the olfactory and gustatory organs of the Dover sole, *Solea*
416 *solea*, during metamorphosis. *Journal of the Marine Biological Association of*
417 *the United Kingdom* **63**, 97-108.

418 Bejarano-Escobar R., Blasco M., DeGrip W.J., Oyola-Velasco J.A., Martín-Partido G.
419 & Francisco-Morcillo J. (2010) Eye development and retinal differentiation in
420 an altricial fish species, the senegalese sole (*Solea senegalensis*, Kaup 1858).
421 *Journal of Experimental Zoology Part B: Molecular and Developmental*
422 *Evolution*. **314B**, 580–605.

423 Blanco-Vives B., Aliaga-Guerrero M., Cañavate J.P., García-Mateos G., Martín-
424 Robles A.J., Herrera-Pérez P., Muñoz-Cueto J.A. & Sánchez-Vázquez, F.J.
425 (2012) Metamorphosis Induces a Light-Dependent Switch in Senegalese Sole
426 (*Solea senegalensis*) from Diurnal to Nocturnal Behavior. *Journal of Biological*
427 *Rhythms* **27**, 135-144.

428 Boeuf G. & Le Bail P. (1999) Does light have an influence on fish growth?
429 *Aquaculture* **177**, 129-152.

430 Bonaldo A., Parma L., Badiani A., Serratore P. & Gatta P.P. (2011) Very early
431 weaning of common sole (*Solea solea* L.) larvae by means of different feeding

432 regimes and three commercial microdiets: influences on performances,
433 metamorphosis development and tank hygiene. *Aquaculture* **321**, 237-244.

434 Cañavate J., Zerolo R. & Fernández-Díaz C. (2006) Feeding and development of
435 Senegal sole (*Solea senegalensis*) larvae reared in different photoperiods.
436 *Aquaculture* **258**, 368-377.

437 Cañavate J., Prieto A., Zerolo R., Sole M., Sarasquete C. & Fernández-Díaz C. (2007)
438 Effects of light intensity and addition of carotene rich *Dunaliella salina* live
439 cells on growth and antioxidant activity of *Solea senegalensis* Kaup (1858)
440 larval and metamorphic stages. *Journal of Fish Biology* **71**, 781-794.

441 Cerqueira V.R. & Brügger A.M. (2001) Effect of Light Intensity on Initial Survival of
442 Fat Snook (*Centropomus parallelus*, Pisces: Centropomidae) Larvae. *Brazilian*
443 *archives of biology and technology* **44**, 343-349.

444 Cobcroft J.M., Pankhurst P.M., Hart P.R. & Battaglione S.C. (2001) The effects of light
445 intensity and algae-induced turbidity on feeding behaviour of larval striped
446 trumpeter. *Journal of Fish Biology* **59**, 1181–1197.

447 Cobcroft J.M. & Pankhurst P.M. (2003) Sensory organ development in cultured striped
448 trumpeter larvae *Latris lineata*: implications for feeding behaviour. *Marine &*
449 *Freshwater Research* **54**, 669–682.

450 Cuvier-Péres A., Jourdan S., Fontaine P. & Kestemont P. (2001) Effects of light
451 intensity on animal husbandry and digestive enzyme activities in sea bass
452 *Dicentrarchus labrax* post-larvae. *Aquaculture* **202**, 317-328.

453 Denson M.R. & Smith T.I.J. (1997) Diet and light intensity effects on survival, growth,
454 and pigmentation of Southern flounder *Paralichthys lethostigma* larvae.
455 *Journal of the World Aquaculture Society* **28**, 366-371.

456 Downing G. & Litvak M.K. (1999) The effect of photoperiod, tank colour and light
457 intensity on growth of larval haddock. *Aquaculture International* **7**, 369-382.

458 Downing G. & Litvak M.K. (2001) The effect of light intensity and spectrum on the
459 incidence of first feeding by larval haddock. *Journal of Fish Biology* **59**, 1566-
460 1578.

461 Fernández-Díaz C., Yúfera M., Cañavate J.P., Moyano F.J. & Díaz M. (2001) Growth
462 and physiological changes during metamorphosis of Senegal sole reared in the
463 laboratory. *Journal of Fish Biology* **58**, 1086-1097.

464 Ferraresso S., Bonaldo A., Parma L., Cinotti S., Massi P., Bargelloni L. & Gatta, P.P.
465 (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.).
466 *BMC Genomics* **14**, 315-337.

467 Fielder D.S., Bardsley W.J., Allan G.L. & Pankhurst, P.M. (2002) Effect of
468 photoperiod on growth and survival of snapper *Pagrus auratus* larvae.
469 *Aquaculture* **211**, 135-150.

470 García-López Á., Pascual E., Sarasquete C. & Martínez-Rodríguez G. (2006)
471 Disruption of gonadal maturation in cultured Senegalese sole *Solea*
472 *senegalensis* Kaup by continuous light and/or constant temperature regimes.
473 *Aquaculture* **261**, 789–798.

474 Geffen A.J., Van der Veer H.W. & Nash R.D.M. (2007) The cost of metamorphosis in
475 flatfishes. *Journal of Sea Research* **58**, 35-45.

476 Grati F., Scarcella G., Polidori P., Domenichetti F., Bolognini L., Gramolini R.,
477 Vasapollo C., Giovanardi O., Raicevich S., Celić I., Vrgoč N., Isajlovic I., Jenič
478 A., Marčeta B. & Fabi G. (2013) Multi-annual investigation of the spatial

479 distributions of juvenile and adult sole (*Solea solea*, L.) in the Adriatic Sea
480 (Northern Mediterranean). *Journal of Sea Research* **84**, 122-132.

481 Henne J.P. & Watanabe W.O. (2003) Effects of light intensity and salinity on growth,
482 survival, and whole-body osmolality of larval Southern founder (*Paralichthys*
483 *lethostigma*). *Journal of the World Aquaculture Society* **34**, 450-465.

484 Howell B.R. & Baynes S.M. (2004) Abiotic factors. In: Culture of Cold-Water Marine
485 Fish (eds. By E. Moksness, E. Kjorsvik, Y. Olsen), pp. 7-27. Blackwell
486 Publishing Ltd.

487 Hubbs C. & Blaxter J.H.S. (1986) Development of sense organs and behaviour in
488 teleost larvae with special reference to feeding and predation avoidance.
489 *Transactions of the American Fisheries Society* **115**, 98–114.

490 Huse I. (1994) Feeding at different illumination levels in larvae of three marine teleost
491 species: cod, *Gadus morhua*, Plaice, *Pleuronectes platessa*, and turbot,
492 *Scophthalmus maximus*. *Aquaculture and Fisheries Management* **25**, 687-695.

493 Jirsa D., Drawbridge M. & Stuart K. (2009) The effects of tank color and light
494 intensity on growth, survival, and stress tolerance of white seabass,
495 *Atractoscion nobilis*, larvae. *Journal of the World Aquaculture Society* **40**, 702-
496 709.

497 Job S.D. & Bellwood D.R. (2000) Light sensitivity in larval fishes: Implications for
498 vertical zonation in the pelagic zone. *Limnology and Oceanography* **44**, 362–
499 371.

500 Lombarte A. & Aguirre H. (1997) Quantitative differences in the chemoreceptor
501 systems in the barbels of two species of Mullidae (*Mullus surmuletus* and *M.*

502 *barbatus*) with different bottom habitats. *Marine ecology progress series* **150**,
503 57-64.

504 Lund I., Steenfeldt S.J. & Hansen B.W. (2007) Effect of dietary arachidonic acid,
505 eicosapentaenoic acid and docosahexaenoic acid on survival, growth and
506 pigmentation in larvae of common sole (*Solea solea* L.). *Aquaculture* **273**, 532-
507 544.

508 Lund I., Steenfeldt S.J., Banta G. & Hansen B.W. (2008) The influence of dietary
509 concentrations of arachidonic acid and eicosapentaenoic acid at various stages
510 of larval ontogeny on eye migration, pigmentation and prostaglandin content of
511 common sole larvae (*Solea solea* L.). *Aquaculture* **276**, 143-153.

512 Lund I., Steenfeldt S.J. & Hansen B.W. (2010) Influence of dietary arachidonic acid
513 combined with light intensity and tank colour on pigmentation of common sole
514 (*Solea solea* L.) larvae. *Aquaculture* **308**, 159-165.

515 Mc Lauren I.A. & Avendano P. (1995) Prey field and diet of larval cod on Western
516 Bank, Scotian Shelf. *Canadian Journal of Fisheries and Aquatic Sciences* **52**,
517 448-463.

518 Monk J., Puvanendran V. & Brown J.A. (2006) Do different light regimes affect the
519 foraging behaviour, growth and survival of larval cod (*Gadus morhua* L.)?
520 *Aquaculture* **257**, 287-293.

521 Mukai Y., Tuzan A.D., Lim L.S. & Yahaya S. (2010) Feeding behavior under dark
522 conditions in larvae of sutchi catfish *Pangasianodon hypophthalmus*. *Fisheries*
523 *Science* **76**, 457-461.

- 524 Mukai Y. & Lim L.S. (2012) Visual thresholds for feeding and optimum light intensity
525 for larval rearing of Asian seabass, *Lates calcarifer* (Bloch). *Aquaculture*
526 *Research* **45**, 188-194.
- 527 Ortiz-Delgado J.B., Iglesias J., Sánchez F.J., Cal R., Lago M.J., Otero J.J. &
528 Sarasquete C. (2012) A morphohistological and histochemical study of
529 hatchery-reared European hake, *Merluccius merluccius* (Linnaeus, 1758),
530 during the lecitho-exotrophic larval phase. *Scientia Marina* **76**, 259–271.
- 531 Otteson D.C. & Hitchcock P.F. (2003) Stem cells in the teleost retina: Persistent
532 neurogenesis and injury-induced regeneration. *Vision Research* **43**, 927-936.
- 533 Padrós F., Villalta M., Gisbert E. & Estévez A. (2011) Morphological and histological
534 study of larval development of the Senegal sole *Solea senegalensis*: an
535 integrative study. *Journal of Fish Biology* **79**, 3-32.
- 536 Pankhurst P.M. & Hilder P.E. (1998) Effect of light intensity on feeding of striped
537 trumpeter *Latris lineata* larvae. *Marine and Freshwater Research* **49**, 363–368.
- 538 Parma L., Bonaldo A., Massi P., Yufera M., Martínez-Rodríguez G. & Gatta P.P.
539 (2013) Different early weaning protocols in common sole (*Solea solea* L.)
540 larvae: Implications on the performances and molecular ontogeny of digestive
541 enzyme precursors. *Aquaculture* **414-415**, 26-35.
- 542 Parra G. & Yufera M. (2001) Comparative energetics during early development of two
543 marine fish species, *Solea senegalensis* (Kaup) and *Sparus aurata* (L.). *The*
544 *Journal of Experimental Biology* **204**, 2175–2183.
- 545 Peña R., Dumas S., Saldivar-Lucio R., García G., Trasviña A. & Hernández-Ceballos
546 D. (2004) The effect of light intensity on first feeding of the spotted sand bass

547 *Paralabrax maculofasciatus* (Steindachner) larvae. *Aquaculture Research* **35**,
548 345-359.

549 Pinto W. (2013) Metamorphosis. In: Larval fish aquaculture (ed. By J.G. Qin), pp.
550 143-166. Nova Science Publisher Inc., New York.

551 Puvanendran V. & Brown A.J. (2002) Foraging, growth and survival of Atlantic cod
552 larvae reared in different light intensities and photoperiods. *Aquaculture* **214**,
553 131-151.

554 Roo F.J., Socorro J., Izquierdo M.S., Caballero M.J., Hernández-Cruz C.M., Fernández
555 A. & Fernández-Palacios H., 1999. Development of red porgy *Pagrus pagrus*
556 visual system in relation with changes in the digestive tract and larval feeding
557 habits. *Aquaculture* **179**, 499–512.

558 Salen-Picard C., Darnaude A.I., Arlhac D. & Harmelin Vivien M.I. (2002)
559 Fluctuations of macrobenthic populations: a link between climate-driven river
560 run-off and sole fishery yields in the Gulf of Lions. *Oecologia* **133**, 380-388.

561 Stuart K.R. & Drawbridge M. (2011) The effect of light intensity and green water on
562 survival and growth of cultured larval California yellowtail (*Seriola lalandi*).
563 *Aquaculture* **321**, 152-156.

564 Stuart K.R. (2013) The effect of light on larval rearing in marine finfish. In: Larval fish
565 aquaculture (ed. By J.G. Qin), pp. 25-40. Nova Science Publisher Inc., New
566 York.

567 Thistle D. (2003) The deep-sea floor: an overview. In: Ecosystems of the World
568 Volume 28: Ecosystems of the Deep Ocean (ed. P.A. Tyler), pp. 5-37. Elsevier
569 Science, Amsterdam.

- 570 Vallés R. & Estévez A. (2013) Light conditions for larval rearing of meagre
571 (*Argyrosomus regius*). *Aquaculture* **376-379**, 15-19.
- 572 Van der Meeren T., Mangor-Jensen A. & Pickova J. (2007) The effect of green water
573 and light intensity on survival, growth and lipid composition in Atlantic cod
574 (*Gadus morhua*) during intensive larval rearing. *Aquaculture* **265**, 206-217.
- 575 Villamizar N., Blanco-Vives B., Migaud H., Davie A., Carboni S. & Sánchez-Vázquez
576 F.J. (2011) Effects of light during early larval development of some
577 aquacultured teleosts: A review. *Aquaculture* **31**, 586-594.
- 578 Yamashita Y., Tanaka M. & Miller J.M. (2001) Ecophysiology of juvenile flatfish in
579 nursery grounds. *Journal of Sea Research* **45**, 205-218.

580 **Figure captions**

581

582 Figure 1. Common sole larvae wet weight (mg) recorded during the trial. Data are given
583 as the mean \pm SD from triplicate treatments. The inset graph shows larval wet weight
584 during the first 19 days of the trial in detail. Different letters denote significant
585 differences among the treatments ($P \leq 0.05$).

586

587 Figure 2. **a)** Gut fullness expressed as the: number of *Artemia* found at 6, 8, 10 days
588 post hatching (dph) in the gut of sole larvae reared under different light intensities ($P \leq$
589 0.05); **b)** Gut fullness of larvae at 13 dph classified into four categories: empty,
590 medium, full and very full. Data are given as the mean ($n = 30$) \pm SD. Different letters
591 denote significant differences among the treatments ($P \leq 0.05$).

592

593 Figure 3. Percentages in the degree of metamorphosis in common sole larvae as a
594 function of light intensity regime. S0) symmetrical left and right eye position; S1)
595 asymmetrical position of the left and right eyes, with the left eye starting to migrate; S2)
596 the migrating eye reaches at maximum the midline of the dorsal surface; S3) the
597 migrating eye can be observed from the right ocular side or migrates within the dorsal
598 side; S4) eye translocation is completed and the orbital arch is visible. $N = 60$ larvae per
599 sample per treatment. Different letters denote significant differences among the
600 treatments ($P \leq 0.05$).

601

602 Figure 4. Histological section of the main sensory organs. A) Retina of 8 dph larva.
603 Large, oval cells, 15 μm in diameter, with a centrally located, hyperchromatic round

604 nucleus are present at the base of the PRL (asterisks), interpreted as retinal stem cells
605 (H&E, bar=15 μm). B) 14 dph larva (H&E, bar=200 μm). Flask-shaped neuromasts
606 formed by several elongated cells are present in the frontal region of the head (inset,
607 H&E, bar=15 μm). C) 25 dph larva. (H&E, bar=200 μm). Numerous taste buds formed
608 by two-three cells and projecting into the lumen of the alimentary canal are visible
609 (inset, H&E, bar=15 μm). D) 51 dph larva. (H&E, bar=200 μm). Olfactory bulbs and
610 olfactory epithelium are well developed in these metamorphosed specimens (inset,
611 H&E, bar=100 μm).

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Table 1

Specific growth rate (SGR) and survival of common sole larvae at the end of the trial.

Protocols	SGR (% day ⁻¹)	SGR (% day ⁻¹)	SGR (% day ⁻¹)	Survival (%)
	from 4 to 12 dph	from 12 to 19 dph	from 19 to 51 dph	
LR-1000	11.79 ± 0.78 ^b	16.16 ± 0.92 ^{ab}	8.37 ± 0.30 ^a	27.4 ± 8.1 ^a
LR-500	12.17 ± 0.16 ^b	14.28 ± 0.44 ^a	8.37 ± 0.35 ^a	43.3 ± 5.7 ^{bc}
LR-50	11.93 ± 0.42 ^b	15.32 ± 0.97 ^a	8.86 ± 0.18 ^a	48.7 ± 1.8 ^c
LR-3	7.38 ± 2.41 ^a	18.17 ± 1.58 ^b	9.82 ± 0.19 ^b	30.4 ± 1.9 ^{ab}

Each value is expressed as the mean ± SD. Columns with different superscript letters for a given value are significantly different ($P \leq 0.05$).

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Table 2. Summary of optimal light intensity thresholds for some cultured marine fish species larvae

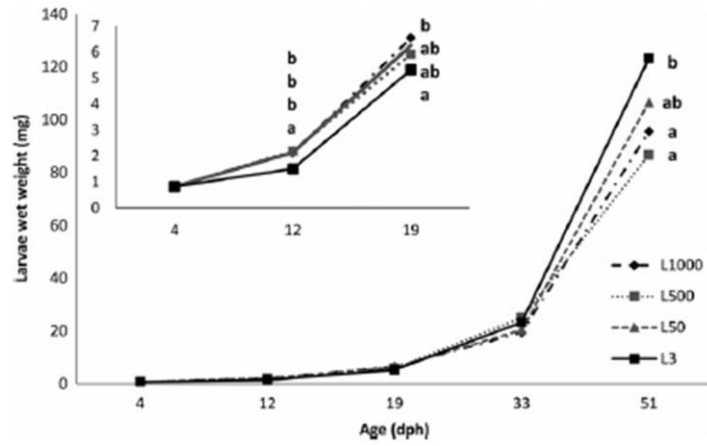
Larvae species	Intensity (Lux)	Age (dph)	References
Southern flounder (<i>Paralichthys lethostigma</i>) (Evseenko)	457 – 1,362	0 - 30	Denson & Smith (1997)
Haddock (<i>Melanogrammus aeglefinus</i> L.)	110	0 - 41	Downing & Litvak (1999)
European sea bass (<i>Dicentrarchus labrax</i> L.)	5 - 400	0 - 21	Cuvier-Péres <i>et al.</i> (2001)
Fat snook (<i>Centropomus parallelus</i>) (Poey)	200 – 1,500	3 - 14	Cerqueira & Brügger (2001)
Atlantic cod (<i>Gadus morhua</i> L.)	2.4 – 56.6	0 - 35	Van der Meeren <i>et al.</i> (2007)
Senegal sole (<i>Solea senegalensis</i>) (Kaup)	200 – 2,000	3 - 20	Cañavate <i>et al.</i> (2007)
White sea bass (<i>Atractoscion nobilis</i>) (Ayres)	300 – 3,000	0-15	Jirsa <i>et al.</i> (2009)
California yellowtail (<i>Seriola lalandi</i>) (Valenciennes)	14,850	2 - 16	Stuart & Drawbridge (2011)
Meagre (<i>Argyrosomus regius</i>) (Asso)	500 – 1,000	1 - 30	Vallés & Estévez (2013)

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655 **Figure 1**



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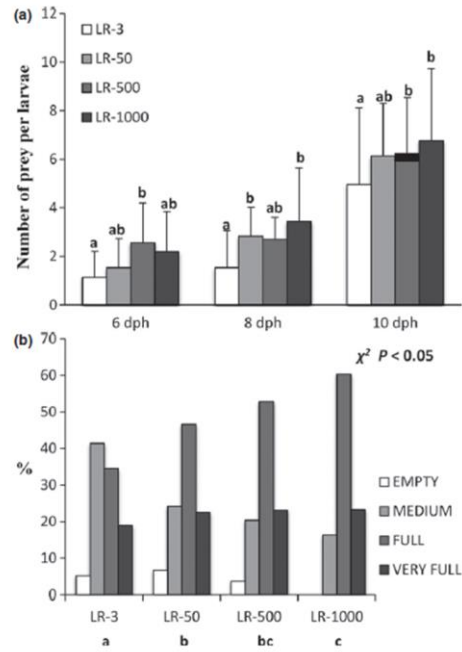
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661 **Figure 2**

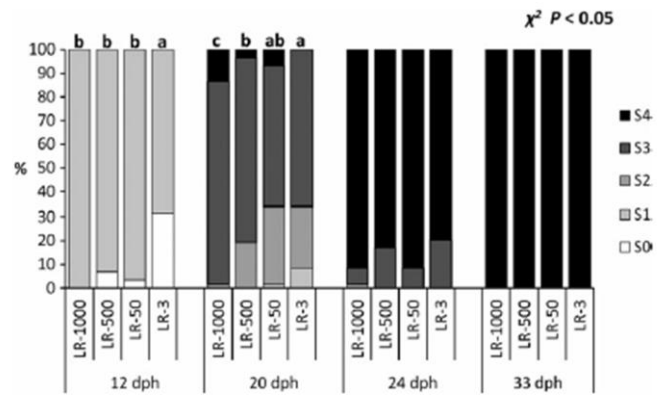


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665 **Figure 3**

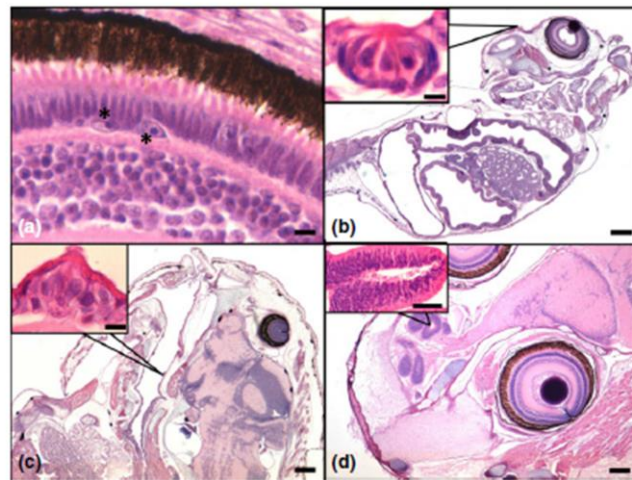


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669 **Figure 4**



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