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1 **Biochemical compounds dynamics during larval development of the carpet-shell**
2 **clam *Ruditapes decussatus* (Linnaeus, 1758): Effects of mono-specific diets and**
3 **starvation**

4

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26 **Abstract**

27 Successful larval growth and development of bivalves depend on energy derived
28 from internal (endotrophic phase) and external (exotrophic phase) sources. The present
29 paper studies survival, growth and biochemical changes in the early developmental stages
30 (from egg to pediveliger) of the clam *Ruditapes decussatus* in order to characterize the
31 nutritional requirements and the transition from the endotrophic to the exotrophic phase.
32 Three different feeding regimes were applied: starvation, and two mono-specific
33 microalgal diets (*Isochysis aff galbana* and *Chaetoceros calcitrans*). A comparison
34 between fed and unfed larvae highlighted the importance of egg lipid reserves,
35 especially neutral lipids, during a brief endotrophic phase of embryonic development
36 (first two days after fertilization). Egg reserves, however, may energetically contribute
37 to the maintenance of larvae beyond the embryonic development. In fed larvae, the
38 endotrophic phase is followed by a mixotrophic phase extending to days 5-8 after
39 fertilization, and a subsequent exotrophic phase. Metamorphosis starts around day 20.
40 The intense embryonic activities are supported by energy derived from lipids, mainly
41 from neutral lipids and the metamorphic activities are supported by energy derived
42 essentially from proteins accumulated during the planktonic phase and depend on the
43 nutritional value of diets. The diet of *I. aff galbana* proves to be more adequate to *R.*
44 *decussatus* larvae rearing. The results provide useful information for a successful
45 production of *R. decussatus* aquaculture.

46

47 **Keywords:** Bivalve larvae; *Ruditapes decussatus*; Biochemical composition; Nutritional
48 phases; Starvation; Diet

49

50

51 **Introduction**

52

53 The clam *Ruditapes decussatus* is widely distributed along the coastal and estuarine
54 areas of Europe and North Africa and has high commercial value. Nevertheless,
55 information on its biology and especially its larval phase is very limited (Chícharo and
56 Chícharo 2001; Beiras and Albentosa 2004).

57 The major morphological changes in bivalve development occur during
58 embryogenesis and metamorphosis. Successful bivalve larval growth and development
59 depend on the energy available during the endotrophic and the subsequent exotrophic
60 developmental phases (Labarta et al. 1999b; Pernet et al. 2004). The endotrophic phase
61 largely corresponds to the embryonic development and is mainly managed by
62 endogenous reserves provided to the eggs during oogenesis (Bayne 1973). The
63 subsequent exotrophic phase, which leads to larval metamorphosis, depends on the
64 value of the diet provided to promote larval growth (Whyte et al. 1990). Both phases
65 are characterized by intense morphogenetic activities, while there is only a small
66 increase in size (Bayne et al. 1975). The transition from the endotrophic to the exotrophic
67 phase seems to be gradual; larvae initiate feeding while still using their yolk reserves.
68 There are only a few published studies on this transitional phase (Lucas et al. 1986;
69 Delaunay 1992; Rico-Villa et al. 2009).

70 The roles of tissue proteins, lipids, and carbohydrates differ among bivalve
71 species. The viability of bivalve larvae is limited by the accumulation and/or utilization of
72 either lipids (Millar and Scott 1967; Helm et al. 1973; Holland and Spencer 1973) or
73 proteins (Bartlett 1979; Rodríguez et al. 1990), but not carbohydrates (Collyer 1957;
74 Holland and Spencer 1973; Bartlett 1979; Gallager and Mann 1986; Gallager et al. 1986;
75 Labarta et al. 1999a), although Haws et al. (1993) suggested that carbohydrates may play

76 a part in the optimal utilization of other reserves.

77 The importance of lipids as an energy reserve in pelagic veliger larvae and the
78 relationship between maximal viability and survival of cultured larvae and the initial lipid
79 (especially neutral lipid) content have been reported (Helm et al. 1973; Holland 1978;
80 Gallagher et al. 1986; Ferreiro et al. 1990). There is also evidence that lipids accumulated
81 during the pelagic larval phase are used as the principal energy substrates during
82 metamorphosis in several bivalve species (Holland and Spencer 1973; Gabbot 1976). In
83 contrast, Bartlett (1979) demonstrated that *Crassostrea gigas* accumulates more energy in
84 the form of proteins than neutral lipids, suggesting that in this species protein is the basic
85 energy reserve during metamorphosis. Similar observations were made by Rodríguez et
86 al. (1990) for *Ostrea edulis*.

87 In the present study we compare fed and unfed *R. decussatus* larvae in order to
88 evaluate the development and duration of the endotrophic/exotrophic transition period,
89 and to understand the selective use of the different biochemical substrates in both absolute
90 and energetic terms during larval development. A better understanding of the species'
91 energy metabolism and nutritional requirements would be relevant to a successful
92 production of *R. decussatus* in aquaculture.

93

94 **Material and methods**

95

96 *Microalgae culture conditions*

97 The microalgae *Isochrysis aff galbana* (T-iso) and *Chaetoceros calcitrans* (C.cal)
98 were reared in 10-l flasks with f/2 medium (Guillard 1975), in a temperature-controlled
99 room at 20±2 °C under continuous illumination. Seawater (salinity = 36±1 ‰) was filtered
100 (0.45 µm), and UV-treated. A continuous aeration was provided to enhance growth and

101 prevent the algae from settling. Algae were harvested when the culture had reached the
102 stationary growth phase. Before being used as food, algal cells were counted with a
103 Coulter Counter TA II, 100 μm , from aliquots of 0.5 ml from each culture.

104

105 *Broodstock conditioning*

106 Adult *R. decussatus* (> 35 mm shell length) were collected in March 2006 from Ria
107 de Aveiro (40°42'N; 08°40'W) (western coast of Portugal). Broodstock clams were
108 conditioned at 20 ± 1 °C for 2 months to speed up their gonad development. Clams were
109 continuously fed a mixture of T-iso and C.cal (5×10^8 cells $\text{ind}^{-1}\text{d}^{-1}$) in a 1:1 ratio in terms of
110 size, the dry weight of algae corresponding to 4 % of the dry weight of the clams' soft
111 tissue (Utting and Millican 1997). The water was enriched with this mixed diet and
112 distributed to the tanks at a flow rate of 0.6 to 0.8 l min^{-1} . Clams were induced to spawn
113 by a rapid increase of temperature from 20 °C to 28 ± 1 °C over a 6-h interval.

114

115 *Experimental design*

116 Oocytes from all females spawned (n=5) were pooled and mixed with sperm (about
117 ten spermatozooids per oocyte) for fertilization. Fertilized eggs were collected on a sieve,
118 washed with filtered seawater and redistributed in a known volume of filtered seawater,
119 subsampled and counted. Three samples of about 50,000 eggs each were taken for
120 biochemical analyses, and a sample of 50 eggs was also taken for diameter measurement.
121 The fertilized eggs were incubated in triplicate 5-l tanks, with filtered UV-irradiated
122 seawater, maintained at 22 ± 1 °C, at a density of 100 eggs per ml with slight aeration. After
123 48 h, the D-larvae were collected on a 30- μm mesh screen and the total number of
124 veligers was calculated based on two 1 ml aliquots. The early D-larvae collected were
125 dispensed at an initial density of 10 ± 2 larvae ml^{-1} in 5-l tanks with natural filtered

126 seawater (0.45 μm) and reared in triplicate under three nutritional regimes: unfed and fed
127 with mono-specific algae (T-iso and C.cal, respectively). Food was added daily to each
128 tank at a rate of 50 cells μl^{-1} . Salinity was 36 ± 1 ‰ and temperature was maintained at
129 22 ± 1 °C. Water was renewed every 2-3 days. At each time, samples were taken from each
130 tank in order to estimate survival and mean shell length, and to detect presence of foot
131 and/or any morphological alterations in the velum. Samples of 5,000 larvae each were also
132 taken for biochemical analyses on days 2, 5, 8, 14, 19 and 23. Remaining larvae were
133 transferred to the tanks.

134 Percent larval survival was determined at each water renewal. Antero-posterior shell
135 length was measured for 50 randomly sampled larvae from each replicate using an ocular
136 micrometer. Linear regressions were fitted to length over larval growth trajectories to
137 determine length growth rates from eggs to metamorphic larvae for each treatment.

138 The presence of a foot was scored to determine larval development status. Larvae
139 that showed a clearly visible foot bulging out of the shell (pediveligers) was considered
140 metamorphosed.

141

142 *Biochemical composition of eggs and larvae*

143 Samples of eggs and larvae for biochemical analyses were rinsed with iso-
144 osmotic ammonium formate (3 % w/v) to remove salt, transferred to Eppendorf tubes,
145 frozen and stored in liquid nitrogen, and then freeze-dried. A micro-analytical fractionated
146 extraction scheme developed by Holland and Gabbott (1971) and Holland and Hannant
147 (1973) was followed for the determination of the contents of biochemical components.
148 Lyophilized samples were homogenized in 500 μl distilled water using a sonicator.
149 Samples were sonicated in an ice water bath for three intervals of 10 s at 20 W each to
150 obtain a thoroughly homogenized sample. Separate samples (200 μl) of the initial

151 homogenate were taken for analyses of total lipids, neutral lipids, proteins, total
152 carbohydrates and free reducing sugars. Total lipid content was extracted by the method
153 of Bligh and Dyer (1959) and taken up in 500 μ l chloroform.

154 Total lipids were determined by the methods of Marsh and Weinstein (1966)
155 using tripalmitin as a standard, and the absorbance was determined at 375 nm. Neutral
156 lipids were determined in the same way as total lipids; 200- μ l samples of neutral lipids
157 in chloroform were dried for 20 min at 100 °C and used for determinations.
158 Phospholipids were determined as the difference between total and neutral lipids.

159 Proteins were precipitated by cold 5 % trichloroacetic acid (TCA) and the
160 precipitate washed in warm 1.0 N NaOH. Protein concentration was assayed by the
161 method of Lowry et al. (1951), modified by Bensadoun and Weinstein (1976) and Hess
162 et al. (1978), at 750 nm using serum albumin as a standard.

163 Hydrolysed and unhydrolysed samples of TCA supernatant were used for the
164 determination of total carbohydrates and free reducing sugars by a modification of the
165 method of Folin and Malmros (1929). The components were quantified with a
166 ferricyanate reduction reaction at 420 nm using glucose as a standard.

167 The organic matter was the sum of proteins, total lipids and carbohydrates. Linear
168 regressions were fitted to organic matter over larval growth trajectories to determine
169 organic growth rates from eggs to metamorphic larvae for each nutritional condition.

170 Energy conversion factors used for lipid, carbohydrate and protein were 35.24,
171 17.16 and 18.00 KJ g⁻¹, respectively (Beukema and De Bruin 1979).

172

173 *Statistical analyses*

174 Differences in survival, growth (shell length, shell length growth rate, organic
175 matter and organic matter growth rate), biochemical composition and energy were

176 tested by analyses of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks,
177 whenever the assumptions of ANOVA failed among days of the same nutritional regime
178 and between regimes. Percentage data were arcsine transformed to normalize variance
179 (Sokal and Rohlf 1981). Multiple pair comparisons among means were performed using
180 the post-hoc parametric Tukey test or the non-parametric Dunn's test.

181

182 **Results**

183

184 *Survival and growth*

185 Survivorship of starved larvae was high till day 5 after fertilization (96.27 ± 1.68
186 %), but then showed a steep decline (Fig 1). On day 23 (last record), 23.21 ± 3.95 % of
187 the larvae (equivalent to $13,000 \pm 2,677$ larvae) were still active. In contrast, the survival
188 of fed larvae showed a rather gradual decrease over the experimental period. This
189 decrease was slightly more pronounced after day 14, when metamorphosis began.
190 Highest survival rates were observed in larvae fed with C.cal (71.98 ± 12.25 %),
191 although these values were not significantly different from those of larvae fed with T-
192 iso (67.00 ± 13.70 %). Significant differences in survival rates were, however, observed
193 between unfed and fed larvae (K–W., $H=12.77$, $d.f.=2$, $P=0.002$).

194 The results for growth (length and organic matter) and the presence of foot in
195 clam larvae under the three nutritional regimes are detailed in Table 1. The diameter and
196 organic matter of eggs (day 0) as well as the initial length and organic matter of the
197 early D-veliger larvae (day 2) were the same for all three treatments. During the early
198 developmental stages (from egg to early D-veliger larva), organic matter remained
199 almost constant (27.48 ± 3.55 ng ind⁻¹ to 29.59 ± 1.65 ng ind⁻¹).

200 The growth rate of unfed larvae was only $0.33 \mu\text{m day}^{-1}$, but significant

201 differences in larvae growth were found among days after fertilization (K–W.,
202 $H=82.45$, $d.f.=7$, $P<0.001$). In terms of organic matter a sudden decrease was observed
203 especially following day 5 after fertilization. When expressed as change in organic
204 matter, negative values for growth rates were obtained (-0.91 ± 0.02 ng ind⁻¹), indicating
205 a progressive decrease in the organic tissue of unfed larvae.

206 In contrast, larvae reared with the mono-specific diets showed an increase in
207 length and also in organic matter. Larvae fed with C.cal had the highest length growth
208 rate (4.95 ± 0.02 $\mu\text{m day}^{-1}$), while larvae fed with T-iso showed the highest organic
209 matter growth rate (16.14 ± 1.30 ng day⁻¹). The percentage of foot presence at day 23
210 was higher in larvae fed with T-iso (75.66 %) than in those fed with C.cal (42.81 %).
211 Significant differences among the three nutritional regimes were observed in length
212 (K–W., $H=908.03$, $d.f.=2$, $P<0.001$), length growth rate (ANOVA, $F=2533.93$, $d.f.=2$,
213 $P<0.001$) and organic matter growth rate (ANOVA, $F=365.81$, $d.f.=2$, $P<0.001$). With
214 respect to organic matter, however, significant differences were found only between fed
215 and unfed larvae (K–W., $H=34.36$, $d.f.=2$, $P<0.001$).

216

217 *Biochemical composition and energy contents*

218 Data on the biochemical composition (proteins, total lipids and carbohydrates) of
219 eggs and larvae are presented in Table 2. Proteins and total lipids were the major
220 biochemical components of early stages of *R. decussatus*. Proteins and total lipids
221 remained almost constant until the early D-veliger stage (13.98 ± 3.39 to 14.77 ± 0.57 ng
222 ind⁻¹, and 8.10 ± 2.40 to 7.34 ± 0.00 ng ind⁻¹, respectively), while carbohydrates nearly
223 duplicated (2.31 ± 0.92 to 4.14 ± 0.14 ng ind⁻¹) during this period. Generally, after
224 embryogenesis and trochophore development (day 2 after fertilization) the above
225 biochemical components tended to increase in fed veligers till day 19, but decreased

226 between days 19 and 23. Larvae fed with T-iso presented the highest increase in all
227 three biochemical components. Protein contents increased from 14.77 ± 0.57 to
228 144.33 ± 11.65 ng ind⁻¹ (T-iso) and 72.35 ± 5.08 ng ind⁻¹ (C.cal), and total lipids from
229 7.34 ± 0.00 to 118.09 ± 8.27 ng ind⁻¹ (T-iso) and 47.22 ± 4.99 ng ind⁻¹ (C.cal).
230 Carbohydrates showed the same tendency: in larvae fed with T-iso their contents
231 increased from 4.14 ± 0.14 to 38.29 ± 4.56 ng ind⁻¹, while larvae fed with C.cal showed
232 irregular variations in carbohydrate contents after day 8.

233 In the unfed culture (see also Fig 2), the protein contents showed a slight initial
234 increase between days 2 and 5, followed by an abrupt decrease to a minimum value of
235 0.37 ± 0.00 ng ind⁻¹ on day 23, which represents a decrease of 27.81 % in terms of total
236 organic matter. A similar situation was observed for total lipids; the level remained
237 constant till day 5 followed by a gradual decline till the end of the experiment (day 2:
238 7.34 ± 0.00 ng ind⁻¹; day 23: 1.72 ± 0.48 ng ind⁻¹), which represents a decrease of 12.94 %
239 in total organic matter. No significant changes were observed in proteins (K-W.,
240 $H=13.12$, $d.f.=4$, $P=0.011$, *Dunn's Method* – $P>0.05$) and total lipids (ANOVA,
241 $F=26.93$, $d.f.=4$, $P<0.001$, *Tukey Test* – $P>0.05$) during the first 8 days after
242 fertilization. Both components presented minimum values at the end of the
243 experimental period (day 23). In contrast, the carbohydrate contents remained nearly
244 constant throughout the experimental period. Significant differences were observed in
245 biochemical composition (proteins - K-W., $H=32.99$, $d.f.=2$, $P=<0.001$; total lipids -
246 K-W., $H=34.03$, $d.f.=2$, $P=<0.001$ and carbohydrates - K-W., $H=30.63$, $d.f.=2$,
247 $P=<0.001$) between unfed and fed larvae.

248 The results for neutral lipids, phospholipids, free reducing sugars and
249 polysaccharides in eggs and larvae under the three feeding regimes are shown in Table
250 3. The main contribution to total lipids came from neutral lipids. During the

251 development from eggs to early D-veliger larvae, a slight reduction in energetic lipids
252 (neutral lipids) was observed (from 7.30 ± 2.43 to 5.89 ± 0.00 ng ind⁻¹) while structural
253 lipids (phospholipids) increased (from 0.08 ± 0.04 to 1.45 ± 0.00 ng ind⁻¹) for all three
254 treatments. The veliger larvae fed with T-iso showed the greatest increase in both
255 neutral lipids and phospholipids. Neutral lipids progressively increased till day 19
256 (106.77 ± 9.21 ng ind⁻¹) with a subsequent decrease till day 23 (60.04 ± 29.46 ng ind⁻¹).
257 Phospholipid contents increased till day 23 when 58.04 ± 27.34 ng ind⁻¹ were attained. In
258 larvae fed with C.cal, neutral lipids showed lower values (day 23: 23.78 ± 6.21 ng ind⁻¹)
259 but the same tendency as in larvae fed T-iso. A rapid increase in phospholipid contents
260 from day 5 to day 8 was followed by a decrease between days 8 and 14, while the
261 contents remained constant between days 14 and 23. In unfed larvae the neutral lipid
262 contents showed a significant decrease from day 5 to day 14 (ANOVA, $F=33.16$,
263 $d.f.=4$, $P<0.001$), i.e. there was a rapid utilization of energetic lipids reserves, while the
264 phospholipid contents did not vary significantly throughout the experiment (ANOVA,
265 $F=0.58$, $d.f.=4$, $P=0.683$) (Table 3 and Fig. 3). These findings give a clear idea of the
266 pattern of neutral lipids utilization (13.18 % of organic matter) during the early
267 development of *R. decussatus*. Significant differences were observed in neutral lipids
268 (K-W., $H=29.90$, $d.f.=2$, $P<0.001$) and phospholipids (K-W., $H=27.58$, $d.f.=2$,
269 $P<0.001$) between unfed and fed larvae. Free reducing sugars and polysaccharides
270 increased in all treatments during development from eggs to early D-veliger larvae.
271 From the earliest shell stage to the pediveliger stage, the free reducing sugar levels in
272 fed larvae increased from 3.35 ± 0.14 to 40.58 ± 9.90 ng ind⁻¹ (day 19) with a subsequent
273 decrease till day 23 (26.74 ± 3.65 ng ind⁻¹) in larvae fed with T-iso. Larvae fed with
274 C.cal presented the same trend, with the highest value recorded on day 19 (27.17 ± 11.02
275 ng ind⁻¹). The polysaccharide contents of fed larvae increased until day 8 and then

276 decreased till the end of experiment, except for the final measurement (day 23) in T-iso
277 fed larvae. In the unfed culture, free reducing sugars remained almost constant over the
278 experimental period and the same tendency was observed for the polysaccharide
279 contents, although a slight decrease was detected following day 14. Significant
280 differences were observed in free reducing sugars (K-W., $H=31.31$, $d.f.=2$, $P<0.001$)
281 and polysaccharides (K-W., $H=29.42$, $d.f.=2$, $P<0.001$) between unfed and fed larvae.

282 Table 4 shows the energy content generated by each biochemical component
283 analyzed and the total energy (KJ ind^{-1}). The main contributions to the total energy
284 came from proteins and total lipids. The energy generated by proteins and total lipids
285 was approximately 40 % and 50 % of total energy, respectively whereas carbohydrates
286 contributed only with about 10 %. During larval development significant differences
287 were observed between unfed and fed larvae in terms of total energy (K-W., $H=28.95$,
288 $d.f.=2$, $P<0.001$) and energy generated by proteins (K-W., $H=28.25$, $d.f.=2$,
289 $P<0.001$), total lipids (K-W., $H=33.28$, $d.f.=2$, $P<0.001$) and carbohydrates (K-W.,
290 $H=23.67$, $d.f.=2$, $P<0.001$) . In starved larvae a gradual decrease in total energy and
291 energy generated by proteins, lipids and carbohydrates was observed, representing a
292 total of 0.51 KJ ind^{-1} consumed, from which more than 48 % (0.24 KJ ind^{-1})
293 corresponded to proteins, 46 % (0.23 KJ ind^{-1}) corresponded to total lipids and about 6
294 % (0.03 KJ ind^{-1}) being provided by carbohydrates. In the fed larvae, from the
295 beginning of the larval period until the onset of the metamorphosis (day 19) there was a
296 gradual increase in energy generated from proteins, total lipids and carbohydrates. It
297 should be noted that from the onset of metamorphosis there was a 25 % and 20 % loss
298 of total energy per individual, in larvae fed T-iso and C.cal, respectively.

299 The energy differentials for each component (KJ ind^{-1}) at each different period of
300 larval development are present in Table 5. During the development from eggs to early

301 D-veliger larvae stage (day 0 to 2) a negative differential was observed for the energy
302 provided by total lipids, indicating that this component was consumed during this
303 process, while during metamorphosis (day 19 to 23) negative differentials were
304 observed for all components. In the larvae fed with T-iso the most important energy
305 contribution to metamorphosis came from both proteins and total lipids. Carbohydrates
306 also contributed, but to a lesser extent. However, in the larvae fed with C.cal the most
307 important energy contribution to metamorphosis came from total lipids.

308

309 **Discussion**

310

311 Larval forms often develop from small eggs with low energy content and are thus
312 presumed to become dependent on exogenous food sources at an early stage of
313 development. Endogenous egg reserves are known to be important for survival and
314 growth throughout embryogenesis until exogenous food sources become available
315 (Ojea et al. 2008). In the present study, larvae of *R. decussatus* survived for an
316 extended period of time without access to algal food. The high survival rate of unfed
317 larvae until day 5 after fertilization (76.78 %) suggests that egg reserves can contribute
318 to the maintenance of larvae beyond the period of embryonic development. Even after
319 23 days of starvation (end of record), about 23 % of the larvae were still swimming and
320 did not show any signs of exhaustion. Apparently, larvae of *R. decussatus*, similarly to
321 those of *C. gigas* (Moran and Manahan 2004), have the capacity to survive without
322 phytoplankton for long periods of time. During periods of low phytoplankton
323 availability, larvae may rely on other external sources of energy for the maintenance of
324 their metabolism until phytoplankton food becomes available and growth can occur.
325 According to Moran and Manahan (2004) and Tang et al. (2005), unfed larvae of *C.*

326 *gigas* and *Meretrix meretrix*, respectively, are not fully dependent upon their
327 endogenous reserves to support maintenance metabolism. In our experiment, larvae
328 were maintained in 0.45- μm filtered seawater and could not have met their energy
329 needs by phytoplankton or any large detrital material. Our results support the idea that
330 *R. decussatus* larvae have complemented their nutritional requirements by alternative
331 sources of energy such as dissolved organic material (DOM) in seawater or bacteria as
332 suggested by several authors (Manahan 1990; Gallager et al. 1994; Gomme 2001). An
333 uptake of dissolved organic materials from seawater has been documented for many
334 species of marine invertebrates (Gomme 2001), and has been suggested to be a
335 potentially important source of energy for larvae (Manahan 1990). Due to larval
336 mortality in the culture tanks, populations of bacteria or heterotrophic protists may have
337 grown in the larval cultures between water changes and might have been used as food
338 by the surviving larvae.

339 Larvae fed with mono-specific diets showed a rather gradual decrease in
340 survivorship with time. This decrease was a little bit more pronounced between days 14
341 and 19, when metamorphosis with major morphological and physiological changes
342 starts and movements and feeding are reduced or inhibited.

343 Larval length growth rates of *R. decussatus* from hatching to metamorphosis
344 obtained in the fed treatments (ranged between 4.93 and 5.27 $\mu\text{m}\cdot\text{day}^{-1}$) were similar to
345 those reported in previous work by our team (Matias et al. 2009) and also by Beiras and
346 Albentosa (2004) and Ojea et al. (2008), suggesting that both mono-specific diets used
347 in the experiment had an adequate nutritional quality. However, significant differences
348 were observed between the two diets in terms of length, length growth rates and
349 organic matter growth rate. The diet of T-iso seems to be more adequate since larvae
350 accumulated significantly more organic matter reserves in their tissues, which has

351 allowed them to overcome more successfully the critical phase of metamorphosis
352 (75.66 % larvae with foot). A decrease in organic matter was observed between day
353 19 and 23 (metamorphosis). A similar decrease in the organic content of larvae during
354 metamorphosis had been cited by Videla et al. (1998) in *Ostrea chilensis*, by Rodríguez
355 et al. (1990) and Labarta et al. (1999a,b) in *Ostrea edulis* and by Moran and Manahan
356 (2004) in *Crassostrea gigas*. The larvae fed with C.cal presented the highest length,
357 suggesting that larvae fed with this microalgae seem to allocate more energy to the
358 biosynthesis of the shell, which might have affected the success of the metamorphosis
359 (42.81 % larvae with foot). However, the percentage of metamorphosed larvae for both
360 diets was higher than those reported by Zine et al. (1998), Ojea et al. (2008) and Matias
361 et al. (2009). During starvation an increase in shell length of the clam larvae was
362 observed. This phenomenon has also been described in other bivalves and is related to
363 an increase in shell mass (Laing and Child 1996). It seems that the biosynthesis of the
364 shell is a priority in the distribution of energy resources, even under extreme nutritional
365 stress. One of the main effects of starvation in invertebrates is a decrease in metabolism
366 down to maintenance levels (Mayzaud 1976; Albentosa et al. 1996). Under such
367 conditions, energy is provided by body reserves and/or the catabolism of tissues. Thus,
368 one of the first consequences of starvation is a loss of organic matter, as this was
369 observed in the present study during the 23 days of food deprivation, especially
370 following day 5 after fertilization. A similar decrease in organic matter has also been
371 observed when the quantity or quality of the food provided is inadequate (Albentosa et
372 al. 2002).

373 In the present study, proteins and total lipids were found to be the most important
374 biochemical constituents of *R. decussatus* larvae, independent of the feeding regime,
375 while carbohydrates (consisting at about 60 % of free reducing sugars and at about 40

376 % of polysaccharides) were present in lower quantities. Similar results have been
377 reported for the early developmental stages of other bivalve species (e.g. Videla et al.
378 1998; Labarta et al. 1999b; Tang et al. 2005; Chaparro et al. 2006). In absolute terms,
379 carbohydrates showed a slight increase in both mono-specific diets, while proteins and
380 total lipids increased considerably after the early stages, reflecting an increased
381 ingestion of particulate food. . It should be noted that the increase for each biochemical
382 component was not continuous, presenting different rates according to the stage of
383 development. Larvae fed with T-iso presented higher total lipids and protein contents
384 than larvae fed with C.cal, however no significant differences were observed between
385 diets in terms of biochemical composition. In unfed larvae, in contrast, a decrease of
386 proteins and total lipids was detected, while carbohydrates remained almost constant.
387 These results suggest that total lipids and proteins are the major energy sources during
388 larval development of *R. decussatus*, unlike the situation in adults where glycogen
389 represents the principal energy reserves (Albentosa et al. 2006). This is also evident in
390 the unfed larvae, in which the catabolism of proteins and total lipids contributed most
391 to the larval metabolism, especially after day 5. Also the high negative energy
392 differentials observed, mainly in proteins and total lipids, in the starved larvae during
393 the whole larval development of *R. decussatus* highlight this evidence. Neutral lipids
394 (energetic lipids) were the most abundant lipid constituents of larvae of *R. decussatus*
395 and the main energy source, since in the mono-specific diets neutral lipids were
396 accumulated in greater amounts than phospholipids after the onset of feeding,
397 indicating that the catabolism of endogenous neutral lipids was rapidly offset by food
398 intake during the nutritional transition. Moreover, in conditions of total starvation,
399 energetic lipids decreased more strongly than structural lipids (phospholipids).

400 In the family Ostreidae, larvae undergoing metamorphosis do not feed, since they

401 have already lost the velum while gills have not yet developed. Thus, larvae in this
402 stage use stored proteins and/or neutral lipids as shown by the decrease in the energy
403 contribution of these components (Ferreiro et al. 1990; Rodríguez et al. 1990; Videla et
404 al. 1998; Labarta et al. 1999a). Some authors have supported the idea that lipids,
405 especially neutral lipids, are the principal energy substrate during larval development
406 and metamorphosis (*e.g.* Holland and Hannat 1974; Ferreiro et al. 1990). However,
407 Rodríguez et al. (1990) stated that proteins were more important than lipids in
408 metamorphosis and suggested the lack of agreement being due to differences in
409 experimental conditions. The results of our experiment conciliate both hypotheses.
410 While lipids (especially neutral lipids) were found to be the main source of energy
411 during embryogenesis to the early D-veliger larvae, proteins are probably the most
412 important substrate during metamorphosis.

413 Whyte et al. (1992) in their work on *Crassadoma gigantea* establish that the
414 larvae best equipped to begin the process of metamorphosis present an energy content
415 of around 6.1-6.3 KJ g⁻¹, while the minimum premetamorphic capacity of the larvae is
416 between 4.5 and 5.0 KJ g⁻¹. Indeed, in our study, the larvae fed with T-iso, with an
417 energy content registered immediately before metamorphosis of 9.95 KJ g⁻¹ were able
418 to successfully begin metamorphosis (75.66 %). On the other hand, the larvae fed with
419 C.cal which presented a value of 3.98 KJ g⁻¹, slightly inferior to the minimum
420 premetamorphic suggested by Whyte et al. (1992), presented considerable
421 lower levels of metamorphosis success (42.81 %). Moreover these results support the
422 fact that the T-iso diet is more adequate for *R. decussatus* larvae rearing than the C.cal
423 one.

424 Our results indicate that eggs reserves could energetically contribute to the
425 maintenance of larvae beyond the embryonic phase. Obviously, the transition from the

426 endotrophic to the exotrophic phase in the early development of *R. decussatus* larvae is
427 a gradual process, with the existence of an intermediate, mixotrophic phase, in which
428 yolk reserves and planktonic particles are used simultaneously by young larvae. The
429 endotrophic phase largely corresponds to the first two days after fertilization. It is
430 followed by a mixotrophic phase from day 2 to approximately days 5-8, after which the
431 larvae are exclusively exotrophic. The mixo- and exotrophic phases involve a period of
432 growth with deposition of the prodissoconch II shell as well as increase in the visceral
433 mass and in velum size. Thereafter, around day 20, metamorphosis occurs, starting with
434 the development of the foot and the primary gill filaments and culminating in larval
435 settlement. This intense morphogenetic activity is supported by energy originating from
436 proteins which are accumulated during the planktonic larval phase.

437 Beyond its contribution to our general knowledge of the biochemical dynamics in
438 the early developmental phases of *R. decussatus*, the present study provides some
439 useful information for hatchery production programs of this clam: 1. Among mono-
440 specific algal diets, T-iso seems to be more adequate than C.cal. 2. Food should not be
441 administered until after day 2 following fertilization (endotrophic phase), and thereafter
442 food supply should be rather moderate until day 8 (mixotrophic phase).

443

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584 **Figures legends:**

585

586 **Fig. 1** Survival (mean \pm SD, n = 6) of *Ruditapes decussatus* larvae reared under three
587 levels of nutrition: starvation and two mono-specific diets (*Isochysis aff galbana* = T-
588 iso, and *Chaetoceros calcitrans* = C.cal)

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590 **Fig. 2** Biochemical composition (mean \pm SD, n = 9) of early developmental stages of
591 the clam *Ruditapes decussatus* reared without food

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593 **Fig. 3** Neutral lipids and phospholipids (mean \pm SD, n = 9) in early developmental
594 stages of the clam *Ruditapes decussatus* reared without food

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596 **Table 1** Length (egg diameter and shell length, respectively; mean \pm SD, n = 150),
 597 larval length growth rate (mean \pm SD, n = 3), organic matter (mean \pm SD, n = 3),
 598 organic matter growth rate (mean \pm SD, n = 3), and presence of foot (%) during the
 599 early development of the clam *Ruditapes decussatus* under three levels of nutrition:
 600 starvation and two mono-specific diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros*
 601 *calcitrans* = C.cal)

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603	Food regime	Days post fertilization	Length ($\mu\text{m ind}^{-1}$)	Larval length growth rate ($\mu\text{m day}^{-1}$)	Organic matter (ng ind $^{-1}$)	Organic matter growth rate (ng day $^{-1}$)	Foot (%)
604		0	58.10 \pm 4.18		27.48 \pm 3.55		
		2	96.71 \pm 5.98		29.59 \pm 1.65		
605		5	100.18 \pm 5.70		35.38 \pm 4.22		
		8	99.18 \pm 4.30		26.59 \pm 5.35		
606	Starvation	10	101.36 \pm 5.60		-		
		12	102.31 \pm 4.45		-		
		14	102.27 \pm 6.05		14.59 \pm 1.02		
607		19	104.53 \pm 6.64		10.38 \pm 1.71		
		23	103.75 \pm 13.70	0.33 \pm 0.09	8.16 \pm 0.63	-0.91 \pm 0.02	0.0
608			0	58.10 \pm 4.18		27.48 \pm 3.55	
		2	96.71 \pm 5.98		29.59 \pm 1.65		
609		5	103.53 \pm 5.02		113.19 \pm 12.95		
	T-iso	8	112.48 \pm 4.69		199.19 \pm 15.24		
		10	119.21 \pm 7.02		-		
610		12	129.81 \pm 8.66		-		
		14	150.14 \pm 10.10		232.78 \pm 26.24		
		19	179.25 \pm 9.72		431.84 \pm 71.31		
611		23	184.03 \pm 9.32	4.69 \pm 0.12	322.17 \pm 7.56	16.14 \pm 1.30	75.66
612		0	58.10 \pm 4.18		27.48 \pm 3.55		
		2	96.71 \pm 5.98		29.69 \pm 2.03		
613		5	104.27 \pm 6.50		80.65 \pm 6.11		
	C.cal	8	134.81 \pm 15.30		96.65 \pm 2.93		
614		10	153.88 \pm 6.46		-		
		12	161.53 \pm 9.99		-		
		14	168.48 \pm 11.64		149.98 \pm 11.83		
615		19	174.39 \pm 13.36		180.98 \pm 15.82		
		23	199.67 \pm 12.19	4.95 \pm 0.02	143.20 \pm 7.78	6.31 \pm 0.31	42.81

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622 **Table 2** Principal biochemical composition (mean \pm SD, n = 9) of early developmental
 623 stages of the clam *Ruditapes decussatus* under three levels of nutrition: starvation and
 624 two mono-specific diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros calcitrans* =
 625 C.cal)

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Food regime	Days post fertilization	Gross biochemical composition (ng ind ⁻¹)		
		Protein	Total lipids	Carbohydrates
Starvation	0	13.98 \pm 3.39	8.10 \pm 2.40	2.31 \pm 0.92
	2	14.77 \pm 0.57	7.34 \pm 0.00	4.14 \pm 0.14
	5	20.22 \pm 3.92	7.24 \pm 0.83	4.36 \pm 0.41
	8	13.49 \pm 6.84	5.58 \pm 0.83	4.29 \pm 0.47
	14	4.75 \pm 1.28	2.82 \pm 1.26	4.03 \pm 0.40
	19	2.39 \pm 1.26	1.72 \pm 0.48	3.51 \pm 0.20
	23	0.37 \pm 0.00	1.72 \pm 0.48	3.31 \pm 0.29
T-iso	0	13.98 \pm 3.39	8.10 \pm 2.40	2.31 \pm 0.92
	2	14.37 \pm 0.00	7.34 \pm 0.00	4.14 \pm 0.14
	5	54.86 \pm 14.13	25.16 \pm 1.91	16.56 \pm 0.92
	8	84.46 \pm 11.48	61.28 \pm 2.66	34.64 \pm 1.03
	14	101.28 \pm 26.23	72.59 \pm 4.14	39.01 \pm 1.73
	19	212.94 \pm 55.97	151.18 \pm 30.41	46.26 \pm 9.47
	23	144.33 \pm 11.65	118.09 \pm 8.27	38.29 \pm 4.56
C.cal	0	13.98 \pm 3.39	8.10 \pm 2.40	2.31 \pm 0.92
	2	14.37 \pm 0.00	7.34 \pm 0.00	4.14 \pm 0.14
	5	36.70 \pm 2.02	21.58 \pm 4.08	19.56 \pm 0.85
	8	27.96 \pm 3.08	46.39 \pm 1.72	20.41 \pm 1.13
	14	68.99 \pm 5.34	53.01 \pm 9.11	18.59 \pm 1.03
	19	85.81 \pm 13.44	56.04 \pm 2.48	32.55 \pm 1.16
	23	72.35 \pm 5.08	47.22 \pm 4.99	23.78 \pm 6.21

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651 **Table 3** Neutral lipids, phospholipids, free reducing sugars and polysaccharides (mean
 652 \pm SD, n = 9) in the early developmental stages of the clam *Ruditapes decussatus* under
 653 three levels of nutrition: starvation and two mono-specific diets (*Isochysis aff galbana*
 654 = T-iso, and *Chaetoceros calcitrans* = C.cal)

Food regime	Days after fertilization	Biochemical composition (ng ind ⁻¹)				
		Neutral lipids	Phospholipids	Free reducing sugars	Po	
Starvation	0	7.30 \pm 2.43	0.08 \pm 0.04	1.76 \pm 0.98		
	2	5.89 \pm 0.00	1.45 \pm 0.00	3.35 \pm 0.14		
	5	5.44 \pm 1.22	1.79 \pm 1.72	3.71 \pm 0.16		
	8	3.65 \pm 1.00	1.93 \pm 1.04	3.64 \pm 0.32		
	14	1.45 \pm 0.74	1.86 \pm 1.04	3.31 \pm 0.16		
	19	0.76 \pm 0.34	0.97 \pm 0.24	3.05 \pm 0.16		
	23	0.76 \pm 0.34	0.97 \pm 0.24	2.99 \pm 0.20		
		0	7.30 \pm 2.43	0.08 \pm 0.04	1.76 \pm 0.98	
T-iso	2	5.89 \pm 0.29	1.45 \pm 0.00	3.35 \pm 0.14		
	5	16.20 \pm 1.84	8.96 \pm 1.57	11.60 \pm 0.78		
	8	44.60 \pm 1.77	16.68 \pm 3.34	24.26 \pm 0.85		
	14	44.98 \pm 13.30	26.61 \pm 18.19	33.01 \pm 0.88		
	19	106.09 \pm 9.21	45.09 \pm 23.55	40.58 \pm 9.90		
	23	60.04 \pm 29.46	58.04 \pm 27.34	26.74 \pm 3.65		
		0	7.30 \pm 2.43	0.08 \pm 0.04	1.76 \pm 0.98	
	C.cal	2	5.89 \pm 0.00	1.45 \pm 0.00	3.35 \pm 0.14	
5		16.06 \pm 1.63	5.52 \pm 4.82	8.08 \pm 0.82		
8		18.26 \pm 8.12	31.50 \pm 13.41	9.84 \pm 0.69		
14		29.57 \pm 5.13	23.44 \pm 9.31	13.36 \pm 1.04		
19		30.81 \pm 4.56	23.99 \pm 3.51	27.17 \pm 11.02		
23		23.78 \pm 6.21	23.44 \pm 8.91	10.49 \pm 1.15		

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658 **Table 4** Energy equivalents of the principal biochemical component (mean \pm SD, n =
 659 9) of the early developmental stages of the clam *Ruditapes decussatus* reared under
 660 three levels of nutrition: starvation and two mono-specific diets (*Isochysis aff galbana*
 661 = T-iso, and *Chaetoceros calcitrans* = C.cal)

Food regime	Days after fertilization	Energy content (KJ ind ⁻¹)			
		Proteins	Total lipids	Carbohydrates	Total energy
Starvation	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.00
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.36±0.06	0.26±0.03	0.09±0.02	0.71±0.00
	8	0.24±0.10	0.19±0.03	0.06±0.01	0.50±0.00
	14	0.09±0.03	0.10±0.04	0.03±0.01	0.21±0.00
	19	0.04±0.02	0.06±0.02	0.01±0.00	0.12±0.00
	23	0.01±0.00	0.06±0.02	0.01±0.00	0.08±0.00
T-iso	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.00
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.99±0.25	0.88±0.07	0.28±0.01	2.16±0.2
	8	1.52±0.21	2.16±0.09	0.59±0.01	4.27±0.3
	14	1.82±0.47	2.56±0.15	0.67±0.01	5.05±0.4
	19	3.83±1.01	5.32±1.07	0.79±0.16	9.95±1.8
	23	2.60±0.21	4.16±0.29	0.66±0.08	7.42±0.2
C.cal	0	0.25±0.06	0.29±0.08	0.04±0.02	0.58±0.00
	2	0.26±0.00	0.26±0.00	0.10±0.00	0.62±0.00
	5	0.66±0.04	0.76±0.14	0.34±0.01	1.76±0.1
	8	0.50±0.06	1.63±0.06	0.35±0.01	2.49±0.0
	14	1.24±0.10	1.87±0.32	0.32±0.02	3.43±0.3
	19	1.41±0.03	2.02±0.06	0.56±0.20	3.98±0.2
	23	1.30±0.09	1.66±0.18	0.25±0.02	3.21±0.2

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664 **Table 5** Energy differentials during larval development of the clam *Ruditapes*665 *decussatus* reared under three levels of nutrition: starvation and two mono-specific666 diets (*Isochysis aff galbana* = T-iso, and *Chaetoceros calcitrans* = C.cal).

Food regime	Days after fertilization	Energy differentials (KJ ind ⁻¹)			
		Proteins	Total lipids	Carbohydrates	Total energetic
Starvation	0-2	0.01±0.00	0.03±0.00	0.06±0.00	0.04±0.00
	2-5	0.11±0.03	0.00±0.03	0.01±0.02	0.09±0.07
	5-8	0.12±0.11	0.06±0.05	0.03±0.00	0.21±0.10
	8-14	0.16±0.13	0.10±0.06	0.04±0.01	0.29±0.09
	14-19	0.04±0.03	0.04±0.04	0.01±0.00	0.09±0.06
	19-23	0.04±0.02	0.00±0.03	0.00±0.01	0.04±0.05
T-iso	0-2	0.01±0.00	0.03±0.00	0.06±0.00	0.04±0.00
	2-5	0.73±0.25	0.63±0.07	0.21±0.01	1.57±0.20
	5-8	0.53±0.46	1.27±0.09	0.31±0.01	2.12±0.50
	8-14	0.30±0.46	0.40±0.07	0.08±0.01	0.78±0.41
	14-19	2.01±1.23	2.77±0.97	0.12±0.16	4.90±1.82
	19-23	1.24±1.08	1.17±0.79	0.14±0.24	2.54±1.65
C.cal	0-2	0.01±0.00	0.03±0.00	0.06±0.00	0.04±0.00
	2-5	0.40±0.04	0.50±0.14	0.26±0.01	1.17±0.16
	5-8	0.16±0.08	0.87±0.11	0.01±0.01	0.73±0.18
	8-14	0.74±0.15	0.23±0.30	0.03±0.02	0.94±0.33
	14-19	0.20±0.13	0.07±0.47	0.24±0.19	0.50±0.79
	19-23	0.11±0.10	0.44±0.08	0.31±0.23	0.85±0.04

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668 The increments represent the difference with the preceding stage.

669 The values in bold type are negative.

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