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1 **Evidence of reproductive cost in the triphasic life history of the red alga *Gracilaria***
2 ***chilensis* (Gracilariales, Rhodophyta).**

3

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20

21 **ABSTRACT**

22 The extent of changes in basic physiological and demographic traits associated with
23 reproduction was investigated in the highly cultivated haploid-diploid red alga,
24 *Gracilaria chilensis*. Sixty individuals bearing vegetative and reproductive fronds
25 collected in the natural population of Niebla (39° 52' S, 73° 23' W), in Chile, were
26 cultivated under control culture conditions. Our results demonstrated that vegetative
27 fronds have a higher survival rate and a better growth rate than reproductive ones
28 whatever the type of individual analyzed (male gametophyte, female gametophyte and
29 tetrasporophyte). Moreover, the reproductive fronds clearly showed a decrease in
30 photosynthetic activity compared to non-reproductive ones. In males and
31 tetrasporophytes the photosynthetic reduction in reproductive individual could be
32 explained by a physical effect of reproductive structure development as well as spores
33 release, disrupting the continuity of the photosynthetic cortical tissues. Translocation of
34 photoassimilates from nearby vegetative tissue or on the previous accumulation of
35 photosynthetic products seems to be a prerequisite for reproductive structure
36 development in this species. Altogether, these results document for the first time in *G.*
37 *chilensis* that reproduction has a strong physiological effect on male, female and
38 tetrasporophyte fronds. This trade-off between reproduction, growth and survival, suggest
39 the existence of reproductive costs in the life history of *G. chilensis*.

40

41

42 **Introduction**

43 The existence of reproductive cost has been demonstrated in various animals
44 (Reznick et al. 2000) and plants (Obeso 2002). The concept of reproductive cost,
45 developed by Fisher (1930) and Harper (1967), provides the conceptual framework of the
46 theory of resource allocation (for review see Bazzaz et al. 2000). This theory predicts the
47 existence of a resource trade-off between reproduction, growth and survival, assuming
48 that reproductive effort imposes a cost on an organism in terms of reduced growth or
49 lower survival (Fisher 1930; Bell 1984; Stearns 1992). Consequently, the study of
50 resource investment in reproduction constitutes a crucial parameter in the understanding
51 of life history strategies of any given species (Harper 1967). According to Bell (1984),
52 reproductive cost is a necessary condition for the optimization of life histories. Indeed,
53 the evolution of life history is constrained by the presence of trade-offs among some of
54 the traits that contribute to fitness (Stearns 1992).

55 In terrestrial plants, such trade-offs have been demonstrated experimentally in
56 limited resources conditions either between sexual reproduction and vegetative growth
57 (Obeso 2002) or between reproduction and survival rate (Rameau and Gouyon 1991). In
58 dioecious plants, studies have shown clear differences in responses among sexes, with
59 females investing more carbon, nitrogen, and other resources in reproduction than males
60 (Obeso 2002). This difference in reproductive effort has been mainly related to the
61 production of seeds, fruits and associated structures (Ashman 1994) and has often been
62 linked with differences in photosynthetic performance (Wheelwright and Logan 2004).

63 In seaweed, cost of reproduction remains poorly documented (for review see De
64 Wreede and Klinger 1990) and has been mainly addressed in Phaeophyceae species in

65 which reproductive organs are clearly differentiated (e.g. receptacles and sporophylles)
66 (Vernet and Harper 1980; McCourt 1985; Ang 1992; Mathieson and Guo 1992; Åberg
67 1996; Zou et al. 2011). While a reproductive cost, like growth reduction or decrease of
68 survival rate, has been observed in various fucoids (McCourt 1985; Ang 1992; Mathieson
69 and Guo 1992; Åberg 1996; Chu et al. 2011), the existence of resource trade-off in algae
70 has been discussed, because reproductive organs are normally pigmented and potentially
71 able to photosynthesis before the release of spores or gametes (De Wreede and Klinger
72 1990). However, the reproductive period coincide generally with the end of growth and
73 can be followed by the senescence and the death of the parental thallus (Zou et al. 2006).

74 In Rhodophyta species, resource trade-off between reproduction and vegetative
75 growth was more difficult to assess because reproductive organs are generally embedded
76 in the cortical tissues of the photosynthetic thallus except for female individuals showing
77 a parasitic-like development of cystocarp on the female thallus after fertilization
78 (Hommersand and Fredericq 1995). In *Gracilaria chilensis* and *G. domingensis*, it has
79 been shown that vegetative growth of female gametophyte decreases after fertilization
80 during the development of cystocarps (Santelices and Varela 1995; Guimarães et al.
81 1999), suggesting that resources were limited. Similarly, the results obtained in the three
82 Ceramiacean species, *Antithamnion nipponicum*, *Ceramium boydenii* and *C. japonicum*,
83 demonstrated that the number and size of spores released depends on the length of the
84 vegetative branch on which cystocarps were produced (Kamiya and Kawai 2002). All
85 these results support the existence of a high photosynthetic investment in reproductive
86 structures of female gametophyte in Rhodophyta. In male and tetrasporophyte
87 individuals, the influence of reproductive structure on survival and growth was rarely

88 assed (but see Santelices and Varela 1995). In these two types of individual, the
89 reproductive structure derives from cortical tissues, and the dual role of the thallus (i.e.,
90 photosynthesis and reproduction) raises questions regarding the potential trade-offs
91 involved in the maintenance of these two functions. Thornber (2006) hypothesized that
92 haploid (male and female gametophytes) and diploid (tetrasporophytes) individuals could
93 differ in vegetative growth, survival and respective investment in reproductive structures.

94 The aim of this study was to analyze the ecophysiological outcomes of the
95 development of reproductive structures in the red seaweed *Gracilaria chilensis* for the
96 three types of individuals (males, females and tetrasporophytes). This species is the most
97 commercially important agarophyte in Chile (Buschmann et al. 2001). We studied the
98 influence of the reproductive status on survival and growth rates as well as basic
99 physiological parameters of interest (i.e., respiration rate, net primary production and
100 pigment content) by comparing reproductively and vegetative fronds of male
101 gametophyte, female gametophyte and tetrasporophyte individuals.

102

103 **Materials and methods**

104 *Species under study*

105 *Gracilaria chilensis* exhibits a typical Polysiphonia-type life cycle with two free-
106 living isomorphic generations. Meiosis occurs in the reproductive diploid individuals
107 (tetrasporophytes) to produce haploid spores (tetraspores). When liberated, tetraspores
108 attach to the substratum, develop perennial holdfasts, and grow into haploid dioecious
109 gametophytic males and females. Male gametes are liberated in the water column,
110 whereas female gametes are retained on the female thallus. After fertilization the

111 carposporophyte (cystocarp) develops on the female haploid plant and the zygote
112 undergoes successive mitoses to produce a gonimoblast from which many thousands of
113 identical diploid spores are formed. When liberated, carpospores attached to the
114 substratum develop into perennial holdfasts, subsequently growing into a tetrasporophyte,
115 completing the complex life cycle. In Chile, natural populations formed by individuals
116 growing from a perennial holdfast and attached to the rocky substratum are encountered
117 between Coquimbo and Raul Marin Balmaceda (30°S and 45°S; Bird et al., 1986). As
118 vegetative fronds share the same morphology, phase and sex of individuals can be
119 recognized only during the reproductive period.

120

121 ***Laboratory experimental design***

122 Individuals of *G. chilensis* were collected at low tide in the natural population of Niebla
123 (39° 52' S, 73° 23' W, Region XIV, Chile) in March 2010. The type of individuals was
124 identified according to their reproductive structures (tetrasporangia for tetrasporophytes,
125 spermatangia for males and cystocarps for fertilized females), using a binocular
126 microscope (SMZ-10; Nikon Co., Tokyo, Japan) (Guillemin et al. 2008). Even if
127 reproductive individuals of *G. chilensis* could be encountered all year round (Meneses,
128 1996), a three-year survey of the Niebla population has shown that the highest percentage
129 of reproductive individuals was encountered during summer (December to April) with at
130 least 85% of reproductive individuals (Guillemin M-L. unpublished data). Twenty
131 tetrasporophytes, 20 females and 20 males, were sampled and cleaned of epiphytes. For
132 each individual, two grams of fresh weight of vegetative fronds and two grams of fresh
133 weight of reproductive fronds were selected to obtain six groups of fronds (vegetative

134 females, males and tetrasporophytes; and reproductive females, males and
135 tetrasporophytes). Following the criteria suggested by Guzmán-Urióstegui et al. (2002),
136 we selected for experimental analyses only the healthy fronds with no damage (i.e.,
137 alteration of the cortical tissue) or discoloration. Only fronds with early stages of
138 reproductive structures were selected to form the three groups of reproductive fronds.
139 The fronds were incubated during two weeks in 1 L Erlenmeyer flask (Schott Duran,
140 Elmsford, NY, USA) of culture medium (Modified SFC culture medium, Correa and
141 McLachlan 1991) aerated with ambient air under controlled photoperiod (12:12 h L:D),
142 photon flux density ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$), temperature ($15 \pm 1^\circ\text{C}$) and salinity (35 PSU). An
143 important spore released was observed during the first 48h for both tetrasporophytes and
144 females. However, a continuous and low spore shading was observed until the end of the
145 experiment for these two types of individuals (spores not counted during our study).
146 Basic physiological parameters and survival rate and growth rates of vegetative fronds
147 were measured on fronds without visible reproductive structure under binocular
148 microscope observation. For reproductive fronds, measures were performed on male and
149 tetrasporophyte fronds showing reproductive structures and, on female fronds, with at
150 least two cystocarps per centimeter. Male and tetrasporophyte reproductive thalli selected
151 presented a continuous and homogenous cover of reproductive structures (100%
152 covering).

153 *Pigments content*

154 Chlorophyll *a* (Chl *a*) was extracted from 10-15 mg fresh weight samples in N, N-
155 dimethylformamide (DMF) following the protocols of Gómez et al (2005). Absorbance
156 values of the supernatant were measured using a spectrophotometer (SUV-2120; Scinco

157 Co., Ltd., Seoul, Korea). Chl *a* concentration were estimated by the equation of Inskeep
158 and Bloom (1985). The phycobilins were extracted from ~ 50 mg fresh weight samples in
159 0.1 M phosphate buffer (pH = 6.5). After centrifugation (30 min at ca. 4500 x g) the
160 concentrations of phycoerythrin (PE) and phycocyanin (PC) were determined
161 spectrophotometrically (SUV-2120; Scinco Co., Ltd., Seoul, Korea) using the equation of
162 Beer and Eshel (1985). For each pigment concentration measurement performed, eight
163 samples of the six frond groups were selected randomly.

164

165 *Metabolic rates and primary productivity*

166 Net Primary Productivity (NPP) and Respiration (R) were measured using the
167 light and dark bottles methodology described in Howarth and Michaels (2000). Fresh
168 fronds of 0.1 ± 0.02 g were placed in Falcon tubes of 50 mL (Becton Dickinson, Cowley,
169 Oxford, UK) filled with filtered seawater sterilized with UV light (QL-40; Rainbow
170 Lifeguard; El Monte, California, USA) and kept in a temperature- and light-controlled
171 incubation chamber ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $15 \pm 1^\circ\text{C}$) for twelve hours. Initial and final
172 dissolved O_2 concentrations were estimated using an YSI 550A oxymeter (YSI Inc.,
173 Yellow Springs, Ohio, USA). No blank correction was applied since oxygen values in
174 light and dark control bottles remained constant. All oxygen values ($\text{mg O}_2 \text{ L}^{-1} \text{ h}^{-1}$) were
175 converted into carbon values ($\text{mg C L}^{-1} \text{ h}^{-1}$) by using a factor of 0.375/PQ (i.e.,
176 photosynthetic quotient). The PQ value was taken as 1 and expressed as a fresh weight
177 basis. Eight replicate measurements were taken for each of the six groups of fronds. After
178 incubation, the algae from the respective bottles were dried at 60°C until a constant
179 weight was reached.

180

181 ***Survival and growth***

182 Fifty frond segments (without apex), 1 cm in length, were excised from each
183 group of fronds (vegetative and reproductive male, female and tetrasporophytes).
184 Separately for each group of fronds, ten segments were randomly selected and placed into
185 a 50 mL culture flask (Orange Scientific, Braine-L'Alleud, Belgium) with filtered
186 seawater without aeration and under low light intensity of $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (5 replicates).
187 Filtered seawater was changed weekly and segments were cleaned using a soft brush.
188 After 50 days, the survival rate was estimated by counting the number of healthy
189 segments (i.e., highly pigmented) and was expressed as a percentage of the original
190 number of segments at the beginning of the experiment. After 60 days, all the bleached
191 fronds fragments had died. The growth rate was estimated after 50 days by measuring the
192 number of new tips formed along the 1cm length frond segment.

193

194 ***Data analysis***

195 Physiological parameters of interest, survival and growth estimated under
196 laboratory conditions were compared using a two-way analysis of variance (ANOVA)
197 and Tukey's Honestly Significant Difference (HSD) tests (STATISTICA 7.0, StatSoft,
198 Tulsa, Oklahoma, USA). The type of individual (female, male and tetrasporophyte) and
199 the reproductive status of the fronds (vegetative or reproductive) were used as fixed
200 factors. ANOVA assumptions (homogeneity of variance and normal distribution) were
201 examined using Cochran and Kolmogorov-Smirnov tests respectively. Transformations

202 preceded the analyses when needed ($\log_{10}(x+1)$ for all three pigment concentrations and
203 Arc Sen $\sqrt{(x/100)}$ for the survival rate).

204

205 **Results**

206 There was a significant interaction between the type of individual (female
207 gametophyte, male gametophyte and tetrasporophyte) and the reproductive status of the
208 frond (vegetative or reproductive) with respect to the concentration of phycoerythrin
209 (PE), net productivity, respiration and growth rates (Table 1). Reproductive females
210 (showing cystocarps) presented the lowest values of pigment content, net productivity
211 and respiration rate of all the fronds measured (Figure 1).

212 The reproductive status of the frond in *G. chilensis* strongly affects all the seven
213 ecophysiological measurements performed during our study ($p < 0.001$, ANOVA Table
214 1, Figure 1). Regardless of sex or phase, vegetative fronds were characterized by a higher
215 pigment content, net productivity, survival and growth rates, than reproductive fronds
216 (Figure 1). Concentration of Chl a and PE were 1.2 to 4.5 times greater in vegetative
217 fronds than in reproductive ones (Figure 1A and B). Net productivity for vegetative
218 fronds was almost twice that of reproductive fronds for all three individual types (Tukey
219 HSD $p < 0.05$, Figure 1D). On the other hand, the respiration rates were roughly similar
220 for all groups of fronds, with the exception of reproductive females, which presented a
221 reduction in the respiration rate (Figure 1E). Striking differences between vegetative and
222 reproductive fronds were observed for survival and growth rates. Indeed, after 50 days,
223 whereas male and female reproductive gametophytes segments had not generated new
224 apex, vegetative segments of 1 cm length (whatever the sex or phase) have generated

225 approximately two new apices (Figure 1F and G). The most conspicuous differences
226 between reproductive and vegetative fronds were observed in female gametophytes and
227 the differences were significant for all the seven measures (Tukey HSD $p < 0.05$, Figure
228 1).

229 There were no consistent differences between the three types of individual except
230 for the phycobilin concentrations (PC), the net productivity and respiration rate (ANOVA
231 Table1). PE and PC were slightly higher in tetrasporophytes than in gametophytes, and
232 the lowest net productivity and respiration rate was seen in female gametophytes
233 (Figure1).

234

235 **Discussion**

236 The results of this study indicate that (1) vegetative fronds of *Gracilaria chilensis*
237 from the natural population of Niebla (Chile) have a higher survival rate and a better
238 growth rate than reproductive ones and (2) the reproductive fronds clearly show a clear
239 decrease in photosynthetic activity compared to non-reproductive fronds.

240 Previous studies in Rhodophyta species demonstrated the effect of reproduction
241 on the photosynthetic activity, such as *Gelidiella acerosa* (Ganzon-Fortes 1999),
242 *Schottera nicaeensis* (Perrone and Felicini 1988) and *Porphyra yezoensis* (Yang et al.
243 2012). These authors suggested that development of new reproductive structures depends
244 on translocation of photoassimilates from nearby vegetative tissue, or on the previous
245 accumulation of photosynthetic products. Yang et al. (2012) demonstrated that complete
246 maturation of the spermatia in *P. yezoensis* was determined by photosynthetic activity at
247 the beginning of the spermatangial development (i.e. the four-cell stage), while in the

248 Chlorophyta *Ulva prolifera*, the formation of the mature sporangia depended directly on
249 the thallus accumulation of photosynthetic products (Gao et al. 2010). In the red algae
250 *Gracilaria cornea*, Guzmán-Urióstegui et al. (2002) pointed out the importance of
251 nutrient supply in the development of cystocarps, and Kamiya and Kawai (2002)
252 demonstrated in Ceramiales species that female reproductive effort depends on the length
253 of the vegetative branch where cystocarps are produced. The dependence of reproductive
254 structure upon nearby vegetative tissues in algae are generally associated to (i) a positive
255 correlation between plant size and fecundity (De Wreede and Klinger 1990; Åberg 1992;
256 Mathieson and Guo 1992; Pfister 1992; Åberg 1996; Zou et al. 2006) and (ii) the
257 existence of a threshold size necessary to achieve reproductive maturity (Gómez and
258 Westermeier 1991; Dyck and De Wreede 2006).

259 Interestingly, in our study, cystocarps, spermatangia and sporangia development
260 was concomitant to a reduction of photosynthetic rate. This was usually associated to a
261 slower growth rate and a higher mortality rate of reproductive fronds in comparison to
262 vegetative fronds. We can hypothesize that the lower net productivity observed in
263 reproductive fronds of *G. chilensis* is directly linked to lower pigment concentration as
264 suggested by Yokoya et al. (2007). In *Gracilaria*, development of reproductive organs as
265 well as the release of tetraspores borne in cortical tissue could affect directly the
266 photosynthetic activity, decreasing the pigment concentration. The negative effect of
267 carposporophyte (cystocarp) on female growth rate has been reported previously in
268 different *Gracilaria* species (*G. sjoestedtii*: Zhang and van der Meer 1988; *G. chilensis*:
269 Santelices and Varela 1995; *G. domingensis*: Guimarães et al. 1999). These results
270 suggest that the development of carposporophyte depend nutritionally on the female

271 gametophyte (Hommersand and Fredericq 1995). Similar relationship showing a negative
272 trade-off between gametophyte growth rate and sporophyte production were also
273 observed in some bryophytes (Bisang and Ehrlen 2002; Rydgren and Økland 2003). Our
274 results corroborate previous studies on *G. chilensis* showing that the reproductive stages
275 have lower growth rate compared to the vegetative stages (Santelices and Varela 1995,
276 Halling et al. 2005) and suggest that reduce growth rate is due to extra energy costs for
277 producing gametes and spores (Kain and Destombe 1995).

278 Generally, the existence of long-term demographic effects of current reproduction
279 on subsequent mortality, growth, or reproduction has remained somewhat unclear in
280 algae, as incongruent results have been reported (De Wreede and Klinger 1990; Ang
281 1992; Dyck and De Wreede 2006). Low reproductive costs were suggested since
282 reproductive structures are potentially capable of photosynthesis (Santelices 1990).
283 Moreover, it has been hypothesized that the comparatively low cost of reproductive
284 structures in relation to the overall energy budget of the plant might minimize the cost of
285 reproduction in algae. In furoid species, Vernet and Harper (1980) estimated that a very
286 low proportion of body weight was invested in gamete production (< 0.4 % for female
287 gametes and < 0.02 % for male gametes). Pfister (1992), suggested that plastic or
288 compensatory responses to the environment may minimize reproduction cost on kelps,
289 and Ang (1992) argued that the failure to detect cost of reproduction with respect to
290 mortality and longevity in *F. distichus* may be due to the modular nature of the plants,
291 where cost occurs at the level of the modules (branches or fronds) rather than at the level
292 of the whole plant (individual). In fact, in *G. chilensis*, Santelices and Varela (1995) have
293 proposed that the negative impact of reproductive structures in female (i.e. cystocarps)

294 could be highly localized. Even though the long-term cost of reproduction at the
295 individual level was not measured in our study, the lower growth and survival of
296 reproductive fronds may have a strong impact on individual fitness. In short-lived
297 seaweeds (or semelparous), release of gametes or spores usually initiates the degradation
298 of the photosynthetic tissues and bouts of sexual reproduction have a clear negative
299 impact on local algal abundance (Clifton and Clifton 1999; Sánchez et al. 2003). In
300 perennial species (or iteroparous), reproduction is generally delayed and depends on the
301 individual size (Åberg, 1992). While fronds constitute the primary photosynthesis organ
302 and only seasonally bear reproductive structure, spores production and individual volume
303 are inextricably linked in *G. gracilis* (Engel et al. 2001). In *G. chilensis*, reproductive cost
304 could have a higher demographic impact in estuaries, where populations reproduce
305 mostly by vegetative fragmentation of fronds than in rocky shore, where individuals are
306 fixed by a perennial holdfast and reproduce solely by spores (Guillemin et al. 2008).

307 In conclusion, the physiological and demographic parameters analyzed in this
308 study allowed the exploration of reproductive cost in red haploid-diploid seaweeds. The
309 results revealed the existence of short-term reproductive cost in *G. chilensis* whatever the
310 type of individual studied (male gametophyte, female gametophyte and tetrasporophyte),
311 providing valuable insight into demographic processes at a local scale. In fact, our results
312 demonstrated that vegetative fronds have a higher survival rate and a better growth rate
313 than reproductive ones. These findings could have useful implications in the management
314 and farming practices of *G. chilensis*. Nevertheless, there is a need to carry out new
315 experimental approach at the population level to estimate the impact of reproduction on
316 individual survival in different local ecological environments, such as muddy or rocky

317 shore. Similarly, since ecological differences have been reported in this species
318 (Santelices and Ugarte, 1990), it is important to understand to what extend environmental
319 variation can influence changes in the energy allocation and trade-offs between
320 reproduction, growth and survival across the geographical range of distribution of *G.*
321 *chilensis* and future studies should be performed in natural populations located in other
322 regions in order to generalized our results.

323

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332

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460 Figure 1: Comparison between reproductive and vegetative fronds of female
461 gametophytes, male gametophytes and tetrasporophytes for: Chl a concentration (A), PE
462 concentration (B), PC concentration (C), net productivity (D), respiration (E), survival of
463 1 cm length segments after 50 days (F) and growth, represented as the number of new
464 apices generated by the 1 cm length segments after 50 days (G). Error bars indicate
465 standard error of the mean and significant differences are represented by different letters
466 (HSD tests, $p < 0.05$).
467