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

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

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ABSTRACT

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

Introduction

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

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

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

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

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

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

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

Laboratory experimental design

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

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

Pigments content

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

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

Metabolic rates and primary productivity

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

Survival and growth

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

Data analysis

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

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

Results

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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