Male gametophyte fragmentation in *Laminaria digitata*: a life history strategy to enhance reproductive success

Christophe DESTOMBE¹,² and L. Valeria OPPLIGER¹,²,³

(1) UPMC Univ. Paris 06, UMR 7144, Equipe “BEDIM”, Station Biologique de Roscoff, 29682 Roscoff, France
(2) CNRS, UMR 7144, Equipe “BEDIM”, Station Biologique de Roscoff, Place Georges Teissier, 29682 Roscoff, France
(3) Departamento de Ecología, Center for Advanced Studies in Ecology and Biodiversity, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Post-code 6513677, Santiago, Chile

Abstract: An understanding of the life history of *Laminaria digitata* is crucial to implementing efficient management practices that can ensure the persistence of the kelp forest. *L. digitata* shows an obligate haplo-diplont heteromorphic life cycle with alternation of a large sporophyte and dioecious microscopic gametophytes. The study of growth and reproduction in culture conditions demonstrated different strategies in male and female gametophytes: Males grew vegetatively and reproduced simultaneously, whereas females stopped growing after reproduction. In addition, we demonstrated that males were able to fragment to give new gametophyte individuals. These results suggest that male and female gametophytes have developed two contrasting reproductive strategies with semelparous females and iteroparous males enhancing the reproductive success of the species.

Résumé : Fragmentation des gamétophytes mâles chez Laminaria digitata : une stratégie pour augmenter le succès reproductif. La compréhension du cycle de vie de Laminaria digitata est primordiale pour assurer le maintien des forêts de laminaires et pour mettre en place les outils de gestion appropriés. *L. digitata* présente un cycle hétéromorphe avec alternance d’individus haploïdes (les gamétophytes microscopiques mâles et femelles) et diploïdes (les sporophytes de grande taille). L’étude de la reproduction et de la croissance des gamétophytes en culture montrent différentes stratégies : les mâles continuent à croître végétativement après la production de gamètes tandis que les femelles stoppent leur croissance végétative après la reproduction. De plus, nous avons montré que les mâles étaient capables de se fragmenter pour donner de nouveaux individus. Ces résultats suggèrent que les mâles et les femelles ont développé deux stratégies opposées : des femelles semelpares qui favorisent la production rapide de nouveaux sporophytes et les mâles itéropares qui assurent la dispersion et augmentent leur succès reproductif.

Keywords: Life history • Gametophyte • Vegetative reproduction • Sex • Resource allocation
Introduction

The study of life history traits such as the mode of reproduction or the mechanisms enhancing successful fertilization are critical to investigate both the ecology of kelps and the persistence of algal stands. The understanding of their life cycle in the field is therefore crucial to design marine protected areas and to implement efficient management practices. However, the kelp population biology is generally poorly known (for review see Schiel & Foster, 2006), mainly because growth and survival of the microscopic gametophyte phase are difficult to study in the field. The relative contribution of the gametophytic phase to the persistence of the kelp forest remains undetermined (Santelices et al., 1995), yet it is necessary to consider the entire life history to understand kelp population dynamics (Begon et al., 2006).

The term ‘life history’ involves all developmental and reproductive events that occur in a population. Sexual reproduction (including meiosis and fertilization) and asexual reproduction (vegetative fragmentation, asexual propagules) are generally the main events in a life history. Russell (1986) considered the life history of an organism to represent an “evolutionary strategy” or a “successful solution” to answer a particular ecological problem. Although the heteromorphic life cycle of *Laminaria digitata* (Hudson) J.V. Lamouroux (as Laminaria flexicaulis Le Jolis) was described one hundred years ago by Sauvageau (1916 & 1918) and Kylin (1916), there are few investigations of the role played by the gametophytes in population dynamics. Most studies have been conducted in the laboratory, and gametophyte stages are generally considered as a “black box” in the field (Schiel & Foster, 2006).

In laboratory conditions, vegetative development of kelp gametophytes is relatively reduced and usually consists of only one or two vegetative cells giving rise immediately to one or two gametes (in *Laminaria digitata* Sauvageau, 1918, in *Lessonia nigrescens* Avila et al. 1985). Consequently, fertilization could occur rapidly after 2 to 3 weeks following spore dispersal (see Dayton, 1985) and most studies assume relatively low gametophyte survivorship in the field (Hsiao & Druehl, 1973; Deysher & Dean, 1986). However, under low irradiance, low nutrients and red light culture conditions, gametophytes are able to grow vegetatively, survive for more than 18 months and reproduce sexually if returned to optimal conditions (tom Dieck, 1993; Yoneshigue-Valentin, 1990). Recently, Carney & Edwards (2010) demonstrated that low-nutrient conditions delay the vegetative development and reproduction of *Macrocystis pyrifera* gametophytes until the return of favourable conditions. These observations suggest that gametophytes may be long-lived organisms that must persist in a semi-dormant stage enabling them to persist as a “microscopic bank” in transitory unfavourable conditions (Chapman, 1986; tom Dieck, 1993; Edwards, 2000; Carney & Edwards, 2006). Moreover, if growth and fragmentation of thalli occurs in the field, gametophytes could become perennial, with profound consequences for the dispersal and reproductive system, producing indefinite numbers of gametes (Kain, 1979). In Phaeophyceae, the presence of reproductive structures on detached seaweed appears to be a common phenomenon (for review, see Macaya et al., 2005). These authors suggest that fragmentation could be an important mechanism for seaweed dispersal. Similarly, we can assume that fragmentation may complement sexual reproduction by increasing the likelihood of finding other suitable gametophytes which in turn would favour reproductive success.

In this study, we analysed the growth rate, the fragmentation and the reproduction of male and female gametophytes of *Laminaria digitata*. The aims of this study were to compare the effect of light quality and agitation of water, on fragmentation and reproduction of gametophytes initially cultivated under low light and nutrients conditions. The objective was to answer the following questions: Are male and female gametophytes equally able to fragment? Do gametophytes grow vegetatively after sexual reproduction? The difference between male and female gametophytes could be fundamental for the understanding of kelp population biology.

Material and methods

Material

*Laminaria digitata* is a perennial species found in upper subtidal rocky shores. Its distribution range extends from Spitsbergen (Svalbard archipelago) in Northern Europe to Southern Brittany (Lüning, 1990). This species shows a haplo-diplont heteromorphic life cycle with an alternation between a large sporophyte (up to 2m) stage and microscopic gametophyte stages. Diploid sporophytes produce haploid spores by meiosis that settle and germinate into microscopic filamentous dioecious gametophytes showing a clear sexual dimorphism (Sauvageau, 1918). Female gametophytes are composed of large cells whereas male gametophytes correspond to highly ramified filaments of small cells. Sexual reproduction is oogamous, female gametophytes produce large immobile gametes (eggs) which, after being fertilized by small gametes (sperms) produce new diploid sporophytes. The encounter of eggs with sperm is under the control of pheromones (Lüning & Müller, 1978; Müller et al., 1979; Maier & Müller, 1990).
The sporophyte does not disperse and overgrows the female gametophyte (Sauvageau, 1918; Müller et al., 1985). However, in culture, unfertilized eggs may develop parthenogenetically, giving rise to abnormally shaped, fragile plants that generally die rapidly (Ar Gall et al., 1996).

**Sampling and culture conditions**

Fragments of 5 fertile sporophytes of *Laminaria digitata* were collected in Northern France at “Les Wardes” (50°55’58.26”N-1°42’02.21”E) close to Wimereux, at the end of January 2008. Mature sori were rinsed with running tap water and sterile seawater immediately after sampling to induce spore release. Fragments (6.3 cm²) from each sporophytes were put into 50 mL Falcon tubes (BD Biosciences, San Jose, CA, USA), each containing a glass slide and sterile seawater. The tubes were carried to the laboratory in darkness and low temperature (ca 10°C). After 12 hours of incubation, spores had settled on the glass slides and sterile seawater was replaced with Provasoli enriched seawater. The 5 Falcon tubes were placed horizontally in culture chambers (12:12 light:dark, 5 µmol·photons·m⁻²·s⁻¹, 15°C), and culture medium was changed every 3 months. Under these conditions, gametophytic individuals remained vegetative. After 9 months of culture, gametophytes from each tubes were detached of the glass slide and isolated individually (one gametophyte per well) in 24 multi-well tissue culture plates with each well containing 2 mL of Provasoli enriched sea-water. A collection of 96 female gametophytes (4 plates) and 96 male gametophytes (4 plates), identified under a binocular microscope according to their sexual dimorphic characteristics, was constituted. The plates were incubated in a growth chamber at 15°C with a photon irradiance of 30-35 µmol·m⁻²·s⁻¹ under 4 different conditions with white and red light, and calm and turbulent environment. For the agitated condition, plates were placed on a shaker platform (“Ping Pong” Rotamax 120, continuous shaking 70 rpm). The culture medium was changed once a week during the first month and then once a month.

**Growth rate**

For each type of gametophytes, individual vegetative growth rate was estimated under different culture conditions during a period of 71 days for the females and 117 days for the males. The first size estimate was made after 5 days of culture in new conditions by measuring the larger diameter of each gametophyte under an inverted microscope (Olympus CKX41). For each of male and female individuals, the growth rate was calculated as follow:

\[
\frac{(\ln(\text{St})-\ln(\text{St-1})) \times 100}{\text{d}}
\]

where St: size at the day n and St-1 at the day m, d: number of days between m and n.

**Fragmentation**

The ability of gametophytes to reproduce vegetatively was tested first by investigating the occurrence of budding on gametophytes and second by counting the number of newly seeded gametophytes per culture well. The number of buds, constituted by small bushy filaments that remain attached at the surface of the main gametophyte (Figure 1), was estimated for each male gametophyte after 117 days of culture by binocular observation. Three classes were defined: No budding, when no evidence of budding was observed on the surface of the gametophyte; Low budding, defined as between 1 and 10 buds per gametophyte; and High budding, when more than 10 buds were observed per gametophyte. The number of newly seeded gametophytes was estimated in each well culture after 117 days by counting under a binocular microscope.

**Gametogenesis and reproductive success**

Gametogenesis was estimated using an inverted microscope (Olympus CKX41) after 10, 15, 20 days of culture. Gametophytes were considered to be fertile if at least 1 male or female reproductive organ was identified. The reproductive success was estimated after 20, 71 and 171 days of culture by counting the number of sporophytes produced per gametophyte.

**Statistical Analyses**

All ANOVAs were conducted using the general linear model procedure of MINITAB (version 13.2 MiniTab Inc. 1994, State College USA). The effect of culture conditions on growth was tested with sex, light and agitation as fixed factors. Tukey’s student range tests were performed for multiple comparisons.

**Results**

**Size of gametophytes**

After 9 months of culture under low light intensity (5 µmol·photons·m⁻²·s⁻¹), gametophytes were spherical and the diameter of female gametophytes was about twice that of male gametophytes (respectively n = 96, x = 496.77 ± 11.72 µm, n = 95, x = 234.88 ± 43.88 µm, Figure 2, Tukey’s pairwise comparisons, P < 0.05).

**Gametogenesis and reproductive success**

All male and female gametophytes were non reproductive after 9 months of culture under low light condition (5
µmol photons m⁻² s⁻¹). After 10 days in new medium and culture conditions, almost all individuals became fertile. Under white light 100% of female gametophytes showed reproductive organs (oogonia) and under red light more than 87% of female were reproductive. The first developments of parthenosporophyte (sporophyte formed without fertilization) were observed after 15 days of culture (sporophytic development occurred on 6% of the females) and after 20 days all females showed sporophytic development. The number of parthenosporophytes per gametophyte after 71 days of culture in high light conditions (photon irradiance of 30-35 µmol.m⁻².s⁻¹) was about twice as high under white light as under red light (for example in agitated conditions the mean number was respectively x = 65.12 ± 43.15 in white light and x = 33.62 ± 9.37 in red light, Tukey’s test, p < 0.05).

**Figure 1. Laminaria digitata.** Male gametophytes after 117 days of culture. A. Spherical gametophyte without bud (scale bar: 20 µm). B. Spherical gametophyte with buds and new seeded gametophytes (scale bar: 20 µm). C. Detail of bud at the surface of gametophyte (scale bar: 5 µm). D. Budding gametophyte producing sperm, (scale bar: 10 µm).

**Figure 1. Laminaria digitata.** Gamétophytes mâles après 117 jours de culture. A. Gamétophyte de forme sphérique sans bourgeonnement (barre d’échelle : 20 µm). B. Gamétophyte de forme sphérique présentant des bourgeonnements en surface (barre d’échelle : 20 µm). C. Détail d’un bourgeonnement à la surface d’un gamétophyte (barre d’échelle : 5 µm). D. Bourgeonnement d’un gamétophyte produisant des spermatozoïdes (barre d’échelle : 10 µm).
Similarly, all male gametophytes were reproductive and released sperm after 10 days of culture in new culture conditions, irrespective of light colour. Surprisingly, after 117 days of culture, 11 male gametophytes (11.5%) showed a few small sporophytes, with a mean number of sporophytes per gametophyte of 1.75 ± 1.52 under red light and 2.5 ± 1.91 under white light.

**Growth rate**

The growth rate depended mainly on the gender of the gametophyte (ANOVA, p < 0.001, Table 1) and on the time of the culture (ANOVA, p < 0.001, Table 1). The mean growth rate of males was about ten times higher than the growth rate of females (respectively x ± SD = 0.00459 ± 0.01298 and x ± SD = 0.00052 ± 0.01649; Figure 3). There was a significant interaction between the factors sex and time (ANOVA, p < 0.001, Table 1). Contrary to males, which showed relatively constant growth rate throughout the experiment, the female growth rate decreased with time, especially after the production of parthenosporophytes (Figure 3). The male growth was higher under white light than under red light, whereas for the females it was the opposite (ANOVA, interaction Sex x Light, p = 0.023, Table 1). Similarly, the interaction between sex and agitation conditions showed that the male growth rate was favoured under agitated conditions (ANOVA, p = 0.001, Table 1).

**Fragmentation**

Vegetative reproduction by fragmentation was solely observed in male gametophytes (Fig 1). At the end of
the experiment (117 days) a total of 67 individuals (69%) showed a differentiation of buds at the surface of the spherical gametophytes (Fig 1). The frequency of buds per gametophyte depends on the conditions of culture ($X^2 = 26.60$, $df = 6$, $p < 0.001$). The highest frequencies were observed under red light (Fig. 4).

The number of newly seeded gametophytes per initial individual was significantly higher in agitated than in calm conditions (ANOVA, $p < 0.001$, Table 2). Indeed, after 117 days of culture, the number of new gametophytes per well was about 6 times higher in shaken than in calm conditions (Fig. 5). Light quality did not affect the occurrence of fragmentation (ANOVA, $p = 0.508$, Table 2).

**Discussion**

These laboratory studies show that, contrary to female gametophytes which stop growing after reproduction, male gametophytes are able to develop vegetatively and reproduce simultaneously. In addition, we found that males were able to fragment to give new gametophyte individuals. This fragmentation mechanism seems to vary among individuals, since in our study more than 68% of the gametophytes produced numerous fragments whereas the others did not fragment at all. This mechanism of fragmentation is favoured under agitated conditions. These results may reflect possible male gametophyte adaptation to enhance sexual reproduction in the field.

Temperature and light are the main abiotic factors influencing the growth of kelp gametophytes (for review see Bartsch et al., 2008). Usually, gametophytes survive and grow in red light at relatively low irradiance levels (Lüning & Neushul, 1978). The extremely low-light requirement of gametophytes is also apparent in their capacity to withstand prolonged periods of darkness (Bartsch et al., 2008). For example, it has been shown that gametophytes were able to survive 12–18 months of darkness in culture and even became reproductive afterwards (Druehl & Boal, 1981; tom Dieck, 1993). In our study, the gametophytes cultivated in low light conditions for 9 months remained sterile as expected whereas the abruptly increasing light intensity and enriched seawater induced reproduction of male and female gametophytes.
irrespective of light color. This result is in agreement with a previous study suggesting that a low increase in light intensity at the optimal temperature can be enough to induce gametogenesis (Bartsch et al., 2008).

Sporophytes development was observed rapidly in our unisexual cultures of female gametophytes, but was also observed (although much later) in about 10% of males. The ability of female kelp gametophytes to give rise directly to sporophyte morphology by parthenogenesis has been reported by numerous authors (for review, see Druhl et al., 2005). In L. digitata these plantelets are generally fragile and never developed beyond a size of 1-2 cm (Ar Gall et al., 1996). Similarly, the direct production of sporophytes through male apogamy has been reported in Alaria crassifolia by Nakahara & Nakamura (1973) and recently in seven different kelp species by Druhl et al. (2005) but again these abnormal sporophytes generally die rapidly in culture (Druhl et al., 2005) and no evidence of parthenogenesis and/or apogamy have been demonstrated in the field.

A sexual dimorphism is generally observed in kelps, in which filamentous thalli of males are usually smaller than those of females (Sauvageau, 1918). The cell diameters are approximately 10 µm in female gametophytes and about half that in males (Kain, 1979). Our results confirmed these observations. Moreover our results showed that females had about twice the size of males after 9 months of culture, suggesting that females grew faster than males in low-light condition. Many dioecious plants display sexual size dimorphism; in trees, males are usually bigger than females, whereas in herbs, females are the larger sex (Obeso, 2002). These trends can be explained by a differential somatic cost of reproduction between the sexes. For example, females need to feed seeds and produce fruits whereas males only produce pollen (Delph, 1999). In kelps, photosynthetic activity is the major physiological process impacting growth and reproduction and thereby is highly relevant per se. Consequently, we can assume that the obvious difference of size between male and female gametophytes might be the result of selection, as larger females may be crucial to producing larger eggs. For organisms that reproduce in sea water, the differences in size between male and female gametes are generally thought to be driven by sperm competition, sperm limitation and postzygotic survival (Levitan, 1996). Likewise, males are usually considered to be limited by fertilization whereas females are limited by resources. Bell (1997) suggested that the big size of the female gamete could have two opposing effects on fitness: larger gametes may give rise to more successful zygotes (and then to a bigger sporophyte), but fewer can be produced from a given mass of tissue. The sexual size dimorphism in kelps could be due to the production of big eggs. Interestingly, whereas the size of female and male gametes is different in kelp, the female reproductive organ (oogonium) and the male reproductive organ (antheridium) produces only one reproductive cell (respectively egg and sperm). Consequently, the number of male and female gametes produced per individual depends mainly on the size of the gametophytes and of their life span. In our study, the number of eggs per female was relatively high (more than 50 per gametophyte). This result sharply contrasts with previous studies showing, in optimal culture conditions, low female fecundity with only one or two eggs per gametophyte in Postelsia palmaeformis (Lüning & Neushul, 1978) and in Laminaria digitata (Cosson, 1978). Although male fecundity was little studied, those estimations show a moderate low number of antheridia per gametophyte and when male and female fecundities were measured at the same time, the spermatozoid/egg (S/E) ratio was relatively low and reached 5 and 14 respectively in Lessonia nigrescens (Destombe unpublished data) and in Laminaria digitata (Cosson, 1978). Studies on the evolution of mating systems in plants suggest that investment in male reproduction (pollen number) is lower in selfing than in out-crossing species (Queller, 1984; Charlesworth & Charlesworth, 1978). Consequently the S/E ratio is considered to be a good indicator of the breeding system in Angiosperms (Cruden, 1977) and more recently in Phaeophyceae (Billard et al., 2005). For example, the mean S/E ratio of Fucus spiralis (self-fertilization species) is 43 whereas it is 400 in F. vesiculosus (out-crossing species). According to these studies, kelps would be rather autogamous species. However, these observations seem to be invalidated by population genetic studies of Laminaria digitata (Billot et al., 2003). The clear differences of growth rate and reproduction between sexes in our study suggest that the reproductive period is more extended in males than in females. Indeed, while males continued to grow and reproduce even after reproduction, growth of females decreased after the production of eggs and finally stopped after the initiation of the parthenosporophyte development. This result suggests that the investment in reproduction is higher in females than in males implying a shorter life span of females compared to males. Consequently, extended observations of the number of gametes throughout the period of male and female reproduction could be necessary to better approximate the S/E ratio in kelps.

The sex ratios of kelps in culture are generally unbiased (close to 1:1) (Sauvageau, 1918; Schreiber, 1930; Cosson, 1978; Oppliger et al., 2010) and correspond to the evolutionary stable allocation (Fisher, 1930; Maynard Smith, 1978). However, in some marine sessile invertebrates, as for example the sponge Rhopaloeides odorabile, male biased sex ratios were observed in natural populations
(Whalan et al., 2007). These authors suggested that skewed sex ratio may be a response to increase fertilization success in benthic environments. In our study we observed that males were able to fragment and to give new male individuals. If this fragmentation mechanism occurs in the field, it will be expected to cause male-biased sex ratio in natural populations.

Since male gametes that have a short life span should disperse locally over very short distances within the boundary layer, we can hypothesis that fertilization involving chemoattractant signals (pheromone) depends mainly upon the geographical proximity of male and female gametophytes (i.e. a few millimeters; Maier & Müller, 1986; Bell, 1997). Consequently, successful fertilization requires that male and female gametophytes recruit at short distances from each other and become mature simultaneously. Male fragmentation increases the number of males in the population and then could enhance fertilization success by decreasing male/female distances. Moreover, by investing in vegetative growth, male gametophytes can increase in biomass that is directly used to disperse fragments. We can expect that male fragmentation might be an efficient way to disperse male gametes similar to pollen in higher plants.

Our studies demonstrated that the fragmentation of male gametophyte in culture depends on light color and on hydrodynamic conditions. Male gametophytes grown in red light continue to grow vegetatively and initiate the development of numerous buds. Water motion stimulated male fragmentation and improved the spreading of newly seeded gametophytes. Recently the effects of water motion on kelp reproduction were studied in *Alaria esculenta* (Gordon & Brawley, 2004). These data suggest that water motion inhibited sperm release by diluting the sperm-releasing pheromone produced by mature eggs. In contrast, these authors noted that spore release increased under agitated conditions due to the mechanical rupture of the sporangium. Similarly, in our study, we assume that water motion might be directly responsible for mechanical splitting of the thallus. The fragmentation of gametophytes could be a system to compensate the inefficiency of pheromone signals in high velocity conditions.

In conclusion, our laboratory study provides the first evidence that there is a tradeoff in the resource budgets between growth and sexual reproduction in kelp female gametophyte. The comparison between sexes exhibits differences in morphology and capacity to fragment. These results suggest that male and female gametophytes present two contrasted reproductive strategies: a semelparous habit may be selected in female gametophytes, ensuring a rapid production of sporophytes, whereas a perennial, iteroparous habit may be selected in male gametophytes. Males would then scatter via new thalli, markedly increasing their reproductive success as well as enhancing the out-crossing in this species. Additional genetic analyses of gametophytes in natural populations using microsatellite markers are needed to test such hypothesis. If this is correct, genetic structure analysis associated to re-colonization experiments in the field should allow detection of male gametophytes with the same genotype.

**Acknowledgements**

We first like to thank Gaspard Delebecq and Marine Robuchon for their technical help and Lucienne Latour for improving the English. We would like to thank Peter von Dassow and two anonymous reviewers for their detailed suggestions and comments improving the manuscript. This study was part of the 3 year ANR project ECOKELP (ANR 06 BDIV 012). L.V. Oppliger was supported by a CONICYT-FRENCH EMBASSY PhD grant. Additional support from the Pôle Mer Bretagne, and the Laboratoire International Associé “Dispersal and Adaptation of Marine Species” (LIA DIAMS) is also acknowledged.

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