Temperature effects on the microscopic haploid stage development of *Laminaria ochroleuca* and *Sacchoriza polyschides*, kelps with contrasting life histories

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Abstract: Kelp forests are one of the most diverse and productive ecosystems worldwide. Global climate change and human exploitation threaten the stability of many of these ecosystems. In this study we compare differences in temperature responses during the microscopic haploid stage development between two kelp species in order to test if the annual *Sacchoriza polyschides* outperforms the perennial *Laminaria ochroleuca* at the northern limit of range distribution of *L. ochroleuca*, in Northern Brittany. Germination and mortality, sex ratio, fecundity and reproduction were measured in culture for the two species and under three different temperatures (10°C, 15°C and 25°C). An effect of temperature was found for all traits except the sex ratio. Both species showed no ability to develop gametophytes at the highest temperature of 25°C and were more tolerant to lower temperatures. *S. polyschides* showed higher germination rate, higher fecundity and lower mortality than *L. ochroleuca* during the period of the experiment. In addition, its gametophytes developed earlier than those of *L. ochroleuca*, a competitive advantage found in all temperature conditions. Germination rate, mortality and fecundity were significantly different between the two species. In addition, the two species showed a structural difference in the development of microscopic stages, with *S. polyschides* gametophytes occupying a larger area, which is suggested to result in a greater adhesion capacity. In conclusion, the microscopic stage of the annual species *S. polyschides* had a significant advantage in fitness compared to the perennial *L. ochroleuca*. This annual opportunistic species may outcompete *L. ochroleuca*, at least in Brittany, the study region, corresponding to its northern limit, in areas where they share habitat.

Résumé : Les forêts de laminaires font partie des écosystèmes les plus diversifiés et les plus productifs au monde. Cependant, leur stabilité est de plus en plus menacée par les changements climatiques et les activités humaines. Le but de cette étude est de comparer la réponse à la température des spores de deux espèces de grandes algues brunes afin de tester si l’espèce annuelle *Sacchoriza polyschides* dont l’aire de répartition s’étant du Maroc jusqu’à la Norvège, est meilleure
Introduction

Changes in climate can strongly alter ecosystems, including shifting species distribution ranges (Spooner, 1950; Fletcher & Farrell, 1999; Steneck et al., 2002; Hiscock et al., 2004; Hampe & Petit, 2005; Müller et al., 2009; Wernberg et al., 2010). In seaweeds, temperature is recognized as the major environmental factor controlling geographic range and depth distribution (Breen, 1988; Izquierdo et al., 2002; Steneck et al., 2002; Müller et al., 2009).

Kelp forests are known to be one of the most diverse and productive ecosystems worldwide (Mann, 1973; Steneck et al., 2002; Wernberg et al., 2010). These large brown seaweeds play a fundamental role as structural ecosystem components, serving as food for herbivores and detritivorous animals, and as shelter and nursery for a variety of species (Steneck et al., 2002; Leblanc et al., 2011). Their canopy reduces the light, creating favourable conditions for shade-adapted species, while by reducing water flow, they also influence sedimentation and erosion rates (Steneck et al., 2002). Finally, kelps are, in general, an economically important human resource, as food, fertilizer in agriculture and animal husbandry, and nutrition for cattle (Braud, 1974; Peteiro et al., 2006, Sousa-Pinto & Araújo, 2006). Kelp extracts are used in dye, textiles, cosmetics and pharmaceuticals, and as a thickener in food preparation (Braud, 1974).

The kelp life cycle consists of a microscopic haploid gametophyte phase, alternating with macroscopic diploid sporophytes. These release meiotic spores that settle on the substrate. Spores germinate and develop into dioecious male and female haploid gametophytes, producing antheridia and oogonia, respectively. After fertilization and syngamy, the diploid zygote originates a new sporophyte (Dayton, 1985). The gametophyte phase and subsequent sporophyte embryos are thought to be part of a “bank of microscopic forms” that will, in the following favourable period, originate the macroscopic sporophytes (e.g., Kinlan et al., 2003, Barradas et al., 2011, also reviewed by Carney & Edwards, 2006).

Kelps have been classified as annual or perennial based only on the macroscopic sporophyte life span. In annual species, sporophytes are only found during a portion of the year. In contrast, perennial species have macroscopic sporophytes with a life expectancy greater than one year and that reproduces for at least two years (varying between 2 and 25 years depending on species; Birkett et al., 1998; Steneck et al., 2002). In both annual and perennial species, the life cycle is completed by microscopic forms of unknown longevity. After the decay of annual sporophytes, survival of microscopic stages during the sporophyte resting season is critical for the maintenance of the population, while in perennial species the survival of microscopic stages may become critical only in case of massive destruction. Such a case was reported in 1992 and 1997/98 in Chile, when the ENSO (El Niño Southern Oscillations) caused a rise in seawater temperature, leading to a decrease in reproduction and recruitment of Lessonia nigrescens Bory de Saint-Vincent 1826 (Martínez et al., 2003). When such events take place, surviving microscopic stages may allow rapid recolonization of the affected areas (Ladah & Zertuche-González, 2007).

Analogous to recolonization of open gaps in a forest, annual species are expected to be the pioneer colonizers, playing the role of opportunistic species as shown by Peteiro et al. (2006), who highlighted that Saccorhiza polyschides (Lightfoot) Batters was the fastest-growing...
alga and was able to colonize newly available space in a biennial culture of _Saccharina latissima_ (Linnaeus) C.E. Lane, C. Mayes, Druelh and G.W. Saunders. Competitive interactions of this nature raise concerns for the resilience of such a complex ecosystem in places where human activity and climate change might increase disturbance levels, causing shifts in species composition. Problems include kelp species unable to recolonize areas along disturbed coasts (e.g. Coleman et al., 2008); or after repeated harvesting at the same location (Engelen et al., 2011) and regression of kelp distributional limits (e.g. Assis et al., 2009; Wernberg et al., 2010).

This study was developed with two species with contrasting sporophyte life spans, but similar geographical and depth distributions: _Laminaria ochroleuca_ De La Pylaie with perennial sporophytes and _Saccorhiza polyschides_ with annual sporophytes. Their upper temperature limits are very close, 25°C for _L. ochroleuca_ and 24°C for _S. polyschides_ (tom Dieck & de Oliveira, 1993). Data for minimum survival temperature were not found in earlier reports. However, it is reported that _S. polyschides_ and _L. ochroleuca_ are exposed to an average winter temperature of 4°C in Norway and 10°C in the English Channel, respectively (Braud, 1974; Norton, 1977; van den Hoek, 1982). Taking this into account, it is expected that _S. polyschides_ has a more efficient development at low temperatures than _L. ochroleuca_.

This experimental work aimed to predict what would be the future composition of the kelp forests in the study area in case of a massive destruction or a rise in water temperature. As such, differences in development and fitness were sought in two species that share this same habitat but have contrasting life histories: _L. ochroleuca_, a perennial species, and _S. polyschides_, an annual. To answer this question, (1) differences in development between the two species were investigated, (2) developmental rates of both species at temperatures within the upper range of natural exposure were estimated, (3) the responses of each species to the highest temperature at their geographical southern limit were assessed and, finally, (4) differences in fertility were quantified.

**Materials and Methods**

**Biological material**

Growth of _L. ochroleuca_ sporophytes is seasonal and usually takes place during the winter/spring. In summer-autumn, blade growth is reduced and spore production is maximum (Lüning et al., 2000). The sporophyte of _S. polyschides_ is annual. The growth of the juvenile blade starts in the spring and maximum size is reached in the summer. In the early autumn, sporophytes reach reproductive maturity. Unlike _L. ochroleuca_, the spores are generated at the base of the frond, which breaks with the first winter storms. Microscopic gametophyte stages develop from the spore, overwinter, and generate macroscopic sporophytes in the following spring (Mann, 1973; van den Hoek, 1982).

_L. ochroleuca_ is a Lusitanian species, found very deep in Azores (Tittley & Neto, 2000) and the Gorringe submerged bank, and in the warm and temperate waters from Morocco to the Portuguese and North-West Spanish coasts, as well as very deep in some areas in the Mediterranean. It can also be found from Brittany (France) to the English and Bristol channels (Braud, 1974; van den Hoek, 1982; Birkett et al., 1998; Bartsch et al., 2008). _S. polyschides_ shares this distributional range, but also extends further north along the South and West coasts of England, Wales, Scotland and Ireland, reaching the west coast of Norway (Norton, 1977; Birkett et al., 1998). The study site is, thus, the northern geographical limit of _L. ochroleuca_ and the central distributional range of _S. polyschides_.

The depth at which kelps are able to live varies widely with the local conditions, particularly temperature (kelp are typically cold-adapted species) and light. Their deeper limit is usually determined by light availability. In clear waters, as the whole light spectrum is able to reach deeper areas, kelps may grow below 35 m. More turbid waters will reduce light penetration and change its spectral composition, preventing the kelp from developing at higher depths (Van Den Hoek, 1982, Birkett et al., 1998).

**Culture**

Reproductive tissues of both _L. ochroleuca_ and _S. polyschides_ were collected at Morlaix Bay (Brittany, France) in July 2009, from depths around 5 m. On the day of collection, the 15 most reproductive individuals of each species were chosen, and divided in three sets with 3 tubes each. This way, all tubes in the same set had spores from the same 5 individuals. After washing the tissue with filtered seawater (SW), sporulation was initiated and lasted 15 to 18 h during which the spores settled on a microscope glass slide placed inside a 50 mL falcon tube filled with filtered SW (Oppliger et al., 2011), on a shaker at 100 rpm.

The following day was considered the first day of culture. The SW was replaced by 0.2 µM Provasoli Enriched Seawater (Provasoli, 1968), with medium changes every five days. One tube from each set was placed at 10°C, 15°C and 25°C. A 12:12 h light:dark photoperiod and photon fluence rate of 30 μmol m⁻² s⁻¹ were used. The experiment lasted 26 days and counts were made on day one, two, and every two days subsequently. Each slide had reference points to ensure that the same area was always studied.
To assess spore development, the following different life history phases were distinguished based on their morphology (Figs 1 & 2): non germinated settled meiospores (Ng), germinated spores with one cell (identified by the presence of a germination tube) (1C), germinated spores with two cells (2C), germinated spores with more than two cells (+2C), male gametophytes (M), immature female gametophytes (Fg), mature female gametophytes (Fm) and female gametophytes with sporophyte (Fs). unicellular or multicellular stages were distinguished as the growth may be influenced by environmental factors, such as temperature (Izquierdo et al., 2002).

**Figure 1.** Overall scheme of spore developmental phases, based on morphology. The non dashed arrows indicate the transition that may occur between observations. Ng: non germinated settled meiospores; 1C: germinated spores with only one cell; 2C: germinated spores with two cells; +2C: germinated spores with more than two cells whose gender was not recognizable yet; M: male gametophytes; Fg: female non mature gametophytes; Fm: Female mature gametophytes; Fs: female gametophytes with sporophyte. The dashed arrow indicates the contribution of the male gametes to originate the sporophyte that is adhering to the female at the Fs stage.

**Statistical analysis**

Frequencies of all stages of development were calculated relative to the total number of spores at day 1. Fecundity and reproductiveness were calculated as follows:

\[
\text{Fecundity} = \frac{\text{Mature females} + \text{Females with sporophyte}}{\text{Total females}}
\]

\[
\text{Reproductiveness} = \frac{\text{Females with sporophyte}}{\text{Total females}}
\]

**Sex ratio**

\[
\text{Sex ratio} = \frac{\text{Total males}}{\text{Total females}}
\]

Frequencies of all stages, germination, death and sex ratio, fecundity and reproductiveness from the two species and under different temperatures were subjected to a general linear model (3-way ANOVA) to test whether they differed significantly between species (fixed factor, 2 levels), temperature (fixed factor, 3 levels) or time (fixed factor, 14 levels: 1, 2, 4, 6, 8, 10... 26). Significance was considered for p-values < 0.05. Pairwise differences for significant interaction terms were analyzed using Tukey tests.

Death and germination rate can only be considered while there are still live forms and spores available for germination, respectively. Thus, to study these parameters, only the days satisfying these conditions for both species and all the temperatures were considered. All statistical analyses were carried out with Minitab (State College, PA, USA).

**Results**

**Germination**

Germination rate varied temporally between species and among temperatures (3-way ANOVA, Species x Time, p < 0.001 and Temperature x Time, p < 0.05, Figs 3 & 4, respectively and Table 1). At day two, *S. polyschides* showed a significantly higher germination rate than *L. ochroleuca* (Tukey test, p < 0.001) and both species had a
higher germination rate compared to the following days (Tukey test, Species x Time, p < 0.001 and p < 0.01, S. polyschides and L. ochroleuca, respectively, Fig. 3). No significant difference was found between days 4 and 6 and between both species (Tukey test, Species x Time, p > 0.665).

On day two, germination rate of both species, combined, was significantly higher at 15ºC (Tukey test, p < 0.001).

Mortality

Mortality differed between species depending on temperature (3-way ANOVA, Species x Temperature, p < 0.001, Table 1). However, this difference was due to the higher mortality at 25ºC of L. ochroleuca showing 4 times higher death rate than S. polyschides (Fig. 5, Tukey test, p < 0.001). For S. polyschides, mortality increased with increasing temperature with 4 times higher death rate at 25ºC compared to 10ºC (Tukey test, p < 0.05), whereas for L. ochroleuca mortality was not significantly different between 10 and 15ºC (Tukey test, p = 0.98), but about 7 times higher at 25ºC (Fig. 5, Tukey test, p < 0.001). No temporal variation was detected (3-way ANOVA, time, p = 0.967). At 25ºC, mortality was complete after spore germination for both species obstructing the formation of gametophytes. Consequently measures such as sex ratio, fecundity and reproductiveness of gametophytes were only analysed for the two lower temperatures.

Table 1. 3-way ANOVA and significance values for the effect of species, temperature, time and interactions between these on development traits

<table>
<thead>
<tr>
<th>Variables</th>
<th>germination rate</th>
<th>mortality</th>
<th>male/female</th>
<th>fecundity</th>
<th>reproductiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.403 ns</td>
<td>0.002</td>
<td>0.133 ns</td>
</tr>
<tr>
<td>Temperature</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.107 ns</td>
<td>0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>Time</td>
<td>&lt; 0.001</td>
<td>0.967 ns</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Species x Time</td>
<td>&lt; 0.001</td>
<td>0.742 ns</td>
<td>0.932 ns</td>
<td>&lt; 0.001</td>
<td>0.346 ns</td>
</tr>
<tr>
<td>Temperature x Time</td>
<td>0.042</td>
<td>0.268 ns</td>
<td>0.153 ns</td>
<td>0.126 ns</td>
<td>0.971 ns</td>
</tr>
<tr>
<td>Species x Temperature</td>
<td>0.092 ns</td>
<td>0.001</td>
<td>0.141 ns</td>
<td>0.745 ns</td>
<td>0.459 ns</td>
</tr>
<tr>
<td>Species x Temperature x Time</td>
<td>0.168 ns</td>
<td>0.650 ns</td>
<td>0.203 ns</td>
<td>0.542 ns</td>
<td>0.998 ns</td>
</tr>
</tbody>
</table>

Figure 3. Germination rate of S. polyschides (Sp) and L. ochroleuca (Lo) over time (combined temperatures). Letters were attributed based on the results of Tukey test, and refer to significant differences between mean values (p < 0.05). Figure 4. Combined germination rate of S. polyschides and L. ochroleuca at 10, 15 and 25ºC at days 2, 4 and 6. Different letters refer to significant differences between mean values. Based on Tukey test (p < 0.05).
Male and Female gametophytes

In both species and for both temperatures, male gametophytes appeared at the same time as female, as such, when the female gametophytes became mature, there were already males present. Gametophytes of *S. polyschides* appeared earlier (day 12 at 10°C and day 10 at 15°C) than for *L. ochroleuca* (day 16 at 10°C and day 12 at 15°C, Fig 6). Sex ratio showed no differences between species or among temperature (3-way ANOVA, species, p = 0.403 and temperature, p = 0.107, table 1), but significant temporal variation was detected (3-way ANOVA, time, p < 0.001). Post-hoc comparisons failed to detected temporal differences (Tukey test, Times per species x Temperature p = 0.074), but the general trend showed increasing sex ratio over time from 0.16 at day 10 to 1.12 (mean of the two species and two temperatures) at day 26, when only gametophytes were present (data not shown).

Fecundity and reproductiveness

Fecundity varied between species over time (3-way ANOVA, Species x Time, p < 0.001, Table 1 & Fig. 7), with higher fecundities for *S. polyschides* than for *L. ochroleuca* from day 18 to day 22 (Tukey test, p < 0.05), after which there were no significant differences (Tukey test, p = 0.218). In addition, fecundities were 1.4 times higher at 15°C than at 10°C (3-way ANOVA, temperature, p < 0.01).

Reproductiveness was 9 times higher at 15°C compared to 10°C (3-way ANOVA, temperature, p < 0.01) and showed temporal variation. (3-way ANOVA, time, p < 0.001), with no interaction between the main effects (3-way ANOVA, Temperature x Time, p = 0.071). No difference between the species was detected (3-way ANOVA, species, p = 0.133)

Gametophyte development

The life cycle differences between *L. ochroleuca* and *S. polyschides* extend to their gametophyte development. Male gametophytes were multicellular for both species. However, the male *S. polyschides* had a larger surface area per volume contacting with the substrate when compared with *L. ochroleuca*. This was mainly due to differences in the arrangements of the cells. *S. polyschides* males have a predominantly linear arrangement of cells, whereas *L. ochroleuca* males are aggregated in a ball-like shape (Fig. 2). Female *S. polyschides* gametophytes developed a multi-cellular structure with large cells, in contrast to *L. ochroleuca*, in which female gametophytes were composed of a single large cell (Fig. 2). Female gametophytes of both species produced only a single sporophyte.

Discussion

Temperature effect

*L. ochroleuca* is known to have a northern distributional boundary caused by the 10°C winter isotherm (van den Hoek, 1982). It has also been reported that this species has a temperature optimum for spore development between 12 and 18°C (Izquierdo et al., 2002). Thus, a better development at 15°C was expected.
Contrarily, the distribution of \textit{S. polyschides} is delimited in the North by temperatures of 3ºC and its spores seem to develop more efficiently at temperature between 5ºC and 17ºC (Norton, 1977). It was thus not expected to find significant variation between 10 and 15ºC. The difference in development we observed for \textit{S. polyschides} between 10 and 15ºC might be due to an adaptation to the local environment. \textit{L. ochroleuca} spores, as well as those of \textit{S. polyschides}, are reported to have a maximum development temperature of 23-24ºC for the sporophytes and 25ºC for the gametophytes (tom Dieck & de Oliveira, 1993; Birkett et al., 1998). Thus, we did not expect a total absence of gametophyte development at 25ºC for either species. A possible explanation for this absence is that ecotype differentiation occurs along the distribution range, as has been described for several kelp species (Bolton & Lüning, 1982; Gerard & Du Bois, 1988; Peters & Breeman, 1993). However, to address the question of local adaptation, more than one site would have to be studied for each species, as was done by Bolton & Lüning (1982) for \textit{Laminaria hyperborea} (Gunnerus) Foslie, \textit{L. digitata} (Hudson) J.V. Lamouroux and \textit{Saccharina latissima}, and by Gerard & Du Bois (1988) for \textit{Saccharina latissima}.

There are comparable examples of local adaptation of kelp populations. However, in such examples, the ecotypes that were described are now recognized as different species. For example, Martínez (1999) reveals the occurrence of different ecotypes characterized by difference in survival of the microscopic stages to temperature, between the kelp \textit{Lessonia nigrescens} located in the Central and Northern part of the Chilean Coast. However, these two ecotypes correspond probably to two different sibling species that have been shown recently to have a disjoint geographical distribution (Tellier et al., 2009). The same was found for \textit{Saccharina latissima} (as \textit{Laminaria saccharina}) see Gerard & Du Bois (1988).

Although these species have roughly similar southern distribution boundaries, \textit{L. ochroleuca} was found to be less adapted to the higher temperature than \textit{S. polyschides}, showing a 4 times higher mortality. In the Strait of Messina (Mediterranean), where the highest temperature of their geographic distribution area is felt, it is reported that these species have different depth distribution, with \textit{L. ochroleuca} found deeper than 30 m and \textit{S. polyschides} being able to grow almost until the surface, thus being exposed to different temperatures (van den Hoek, 1982). Van den Hoek (1982) proposed two explanations for this difference in depth distribution: one was that \textit{L. ochroleuca} was forced to occur at higher depths because of occasional increase of water temperature over 23ºC. Another or additional possibility is that it is a more shade tolerant species. The precise distributional patterns of these species support the former hypothesis. Along the Portuguese coast, \textit{L. ochroleuca} is now only found along the northern to central regions, with \textit{S. polyschides} extending further south (Assis et al., 2009). Indeed, reports of occurrence of \textit{L. ochroleuca} further south are mainly very deep populations, occurring below 40 m depths (e.g., Azores, Gorringe bank).

Species with the same geographical boundaries might be delimited by different factors and / or in different life
phases (Breeman, 1988). As such, one of these kelp species could, for example, be delimited by its winter growth requirements and the other by summer reproduction limitations (Breeman, 1988; Birkett et al., 1998). In addition, kelp sporophyte’s heat tolerance varies along the year (Lüning, 1984).

No significant effect of temperature on sex-ratio was detected in this study while temperature-dependent sex ratio was reported in several kelp species (in Laminaria religiosa: Funano, 1983; L. variegata: Nelson, 2005 and Lessonia nigrescens complex: Oppliger et al., 2011). The authors suggested that under favorable conditions, mortality will be at a minimum and sexes will be present in equal proportion, while under stress conditions the more vulnerable sex will suffer the higher mortality suggesting a difference in sex ratio. More experiments are needed to carefully address this question since interestingly the two study species might respond differently to temperature. Although not significant, a slight tendency showing an increased of males in L. ochroleuca and an increase of females in S. polyschides with increasing temperature from 10 to 15°C was reported in our study.

Differences between the two species development

Following the classical ecological succession model, S. polyschides could be considered as a pioneer and opportunistic species that is able to colonize rapidly the open gap in a dense kelp forest, as supported by its initial higher germination rate. The difference in gametophyte morphology following spore germination should reflect such a colonizing ability. Indeed, the demographic dynamics of an annual kelp species is heavily dependent on the survival of microscopic stages during the winter. The morphological characteristic of the S. polyschides gametophyte may correspond to a higher adhesion capacity than L. ochroleuca. The hypothesis is that a bigger surface area provides a superior adhesion capacity, making S. polyschides more resistant to the winter storms. Although no information was found about the life expectancy of these forms, due to the difficulties of studying them in the field, one hypothesis may be that this higher adhesion capacity might allow the gametophytes to remain viable for more than one year, facilitating fast recruitment.

It was reported (Izquierdo et al., 2002) that the number and arrangement of cells in the L. ochroleuca gametophytes can vary with temperature. This was not observed here.

Our results reveal differences in fecundity responses through time between the two species, which may reflect differences in reproductive strategies. The rapid and higher fecundity of female gametophytes (50% after day 16 of culture) in S. polyschides compared to the lower and progressive fecundity curve observed in L. ochroleuca suggests (1) faster growth rate of the female gametophyte and (2) better synchronization of reproduction in S. polyschides, which should minimize the likelihood of eggs not being fertilized. A possible explanation for the difference between these two species responses is a mal-adaptation of L. ochroleuca to environmental conditions at its northern geographical distribution boundary, setting its distributional limit and rendering it more vulnerable to local extinction.

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