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Running head: Stress response in *Laminaria digitata* gametophytes

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Summary

Given the growing body of evidence on the general decline of kelp beds worldwide, it is crucial to understand the physiological response of kelp gametophyte stages to environmental parameters. We investigated the physiological response of gametophytes to light and temperature in two populations of *Laminaria digitata* occurring in two contrasting environments along the French coast of the English Channel. Results indicate that gametophytes of both populations were highly tolerant to high light through an efficient down-regulation of photosynthesis triggered by the activation of the xanthophyll cycle. Temperature increases promoted photosynthesis and the photosystem II showed high resistance to short-term exposure to high temperatures currently encountered in the field. Gametophytes from the two sites displayed some differences in their pigment contents and photosynthetic characteristics, but low replication size and difference in time of sampling did not permit to confirm a potential local adaptation to the light conditions at each site, as observed in previously published results on adult sporophytes. Gametophytes of *L. digitata* appeared to be resistant to irradiation and temperature conditions currently experienced in the field, confirming their role in persistence of kelp species under stressful environmental conditions.

Keywords: Phaeophyceae, Photosynthesis, Xanthophyll Cycle, Stress, Phenotypic Plasticity, English Channel
Introduction

Kelps are major structural components of the cold temperate and boreal coastal communities (Lüning, 1990). They form a highly productive habitat (Mann, 1973) that harbours a rich biodiversity (Christie et al., 2003). The ecological role of kelp is threatened by their expected vulnerability to changes in the physical environment caused by rapid climate change (Wernberg et al., 2011a; Raybaud et al., 2013) or extreme events (Wernberg et al., 2013), especially near range edges, where populations are at the front line of environmental change (Wernberg et al., 2011b).

The persistence of kelp forests mainly depends on the recruitment, growth, competition and the physiological tolerance to environmental factors of the microscopic stages (Reed & Foster, 1984; Ladah & Zertruche-Gonzales, 2007; Matson & Edwards, 2007), which are the crucial phases of the complex heteromorphic life cycle of kelp (Sauvageau, 1915). Gametophytes and embryonic sporophytes can delay their development and reproduction for several months (Carney, 2011) until favourable conditions occur and therefore promote rapid recruitment in the best conditions (Edwards, 2000).

Kelp microscopic stages are subject to environmental conditions that differ from those experienced by the macroscopic sporophytes (Reed & Foster, 1984; Martinez & Santelices, 1998) and can therefore have different physiological optima and tolerance levels (Hanelt et al., 1997; Altamirano et al., 2004; Matson & Edwards, 2007). Even under similar environmental conditions, the response of haploid stages differs from those of diploid stages among various taxa of macroalgae (Roleda et al., 2008; Wang et al., 2011). Hence, in the context of environmental change, enhancing the understanding
of the physiological tolerance of kelp must incorporate the study of their multiple life-
stages, including their microscopic phases (Harley et al., 2012; Wernberg et al., 2012).

Among the potentially adverse environmental conditions, light and temperature can
greatly affect the development and survival of microscopic stages and their vulnerability
generally determines the ecological success of the species (Bartsch et al., 2008).
Extremely high light has been shown to be adverse for the development and viability of
post-settlement stages (gametophytes and embryonic sporophytes) (Lüning & Neushul,
1978; Fetjek et al., 2011). Likewise, extreme high temperature events (such as El Niño
events) have been shown to affect the reproductive success of gametophytes (Ladah &
Zertruche-Gonzales, 2007; Oppliger et al., 2012).

The photosynthetic apparatus is one of the main targets of these abiotic stressors
(Walters, 2005). Rapid fluctuation of light and temperature can cause disruptive stresses
(Davison & Pearson, 1996) and affect the survival of kelp gametophytes. Drastic and
rapid changes in light exposure and temperature are frequently experienced within a
single day (Gevaert et al., 2003; Delebecq et al., 2011) and especially during sunny
spring tides.

Therefore, the ability to withstand stressful conditions and to recover from them is
crucial for preventing damage to the photosynthetic apparatus and maintaining
sufficient photosynthetic performance. After the onset of stressful conditions, the
regulation of energy absorption and utilization is essential (Raven & Geider, 2003).
Photoinhibition is the down-regulation of photosynthesis, whose extent is determined by
the balance between the rate of photodamage and the rate of repair of photosystem II
(PSII) (Takahashi & Murata, 2008). Photoinhibition has been observed in the field on
macroscopic sporophyte stages (Gevaert et al., 2003; Delebecq et al., 2011), in the laboratory on zoospores (Roleda, 2009) and in the gametophytic and embryonic sporophyte stages of kelp (Hanelt et al., 1997; Altamirano et al., 2004). Factors other than light, such as temperature, can accelerate photoinhibition by altering the PSII repair mechanisms (Takahashi & Murata, 2008). Toxic active derivatives of oxygen (oxygen radicals), byproducts of photosynthesis, can also be over-produced under adverse environmental conditions (when exceeding the scavenging potential of cells) and can alter the biological integrity of cells (Ledford & Niyogi, 2005; Allakverdiev et al., 2008).

To cope with excess light, energy is dissipated in the form of heat to rapidly regulate light harvesting; this mechanism is widespread in photoautotrophs (Raven & Geider, 2003). Increased thermal energy dissipation of excess light involves the xanthophyll cycle in brown algae, which plays a major role in the fast-dynamic acclimation to change in light, temperature and desiccation in macroscopic sporophytes (Fernandez-Marin et al., 2011). However, the implication of xanthophyll cycle in the photoprotection process in kelp gametophytes has only been mentioned (Hanelt et al., 1997; Altamirano et al., 2004), without being clearly demonstrated.

*Laminaria digitata* Hudson Lamouroux (1813), a kelp species of high ecological and economical value, has been shown to be retreating from several sites along the French coasts (Arzel, 1998; Davoult et al., 2011), sparking research to determine the reasons for this decline. Physical stress and environmental changes may contribute to a reduction in the fitness of the gametophytic developmental stages of *L. digitata*. In this study, we tested the sensitivity of *L. digitata* gametophytes to changing photon flux density and temperature. The investigations were carried out on two populations of *L.*
digitata along the French coast of the English Channel inhabiting contrasting environmental conditions (Delebecq et al., 2013). The differences in local environmental conditions may result in different sensitivities among the populations that need to be taken into account in understanding the effect of environmental factors on organisms. In this study, we also measured the role of the xanthophyll cycle in the non-photochemical quenching in gametophytes of L. digitata in response to excess light.

Materials and Methods

Study site

A complete description of the two sites and environmental conditions during experiments is given in Delebecq et al., (2013); consequently, we describe only the main characteristics of both sites here. We collected the seaweed material from two populations of L. digitata, growing in the upper subtidal zone (0-1 m) of a rocky shore in Roscoff (48°5′N, 3°6′W) and in Wissant (50°5′N, 1°4′E), located in the western and eastern part of the English Channel, respectively. The sites were selected for their large kelp stands and also for their contrasting environmental conditions. Light attenuation (m$^{-1}$) of photosynthetically active radiation (400–700 nm) ranged from 0.09 to 0.57 m$^{-1}$ in Roscoff and from 0.19 to 0.96 m$^{-1}$ in Wissant (Delebecq et al., 2013) due to high turbidity in the eastern English Channel. Seawater surface temperature displays high seasonal variation at both sites, with a slightly broader annual temperature range in Wissant, from 5°C in winter to 20°C in summer, than in Roscoff, from 8°C in winter to 17°C in summer, due to the influence of the North Atlantic Ocean and depth of the
continental shelf. Environmental conditions in the two sites at the time of collection and
1 month prior to sampling are given in Table 1.

Sampling and culture conditions

Fertile sporophytes of *L. digitata* were collected at low tide in November 2008 in
Roscoff, and in February 2009 in Wissant. *L. digitata* is reproductive most of the year in
Roscoff, but the main spore/gamete release events generally occur in August-September
and November-December in Northern Brittany (Arzel, 1998). In Wissant, *L. digitata* is
reproductive at the end of winter and throughout spring. Mature sori were cut, cleaned,
and dried at 10°C and in the dark for several hours. Sori were subsequently washed with
distilled water and sterile seawater, and placed in 50 mL Falcon tubes (BD Biosciences,
San Jose, CA, USA), filled with sterile Provasoli enriched seawater (PES) (Provasoli,
1968), and maintained overnight in the dark on a rotary shaking table to induce
zoospore release. Zoospores in suspension were checked with inverted-light microscope
to ensure that zoospores were viable, before being placed in Petri dishes (BD Falcon,
Franklin Lakes, NJ, USA). Zoospores were allowed to settle and developing
gametophytes were cultured in thermostatic chamber at 10°C under an irradiance of 35
µmol photons m⁻² s⁻¹ (photon flux density, PFD, 400-700 nm), produced by fluorescent
tubes (L8W/840, cool white, Osram, Germany) in a light:dark cycle 12:12 h. PFD was
measured with a cosine-corrected quantum sensor (Li-192SA, LiCor, Lincoln,
Nebraska, USA), connected to a data logger (Li 1400, LiCor, Lincoln, Nebraska, USA).
The medium was changed once a week. Experiments started approximately after 1
month of cultivation when the density was high enough for fluorescence measurements.
The density of the gametophytes was 4711 ± 1140 ind m⁻² for Roscoff (43 ± 3% cover)
and 3600 ± 691 ind m⁻² for Wissant (41 ± 7% cover). Gametophytes were composed of
few cells and arranged as homogenous thick layers at the bottom of the Petri dishes.

Gametophytes were isolated from three different parents at each site (n = 3) and were cultivated separately to ensure independent replicates.

**Oxygen production**

Dark respiration (\(R_d\)) and net oxygen production (NP) was measured polarographically at culture temperature (10°C), using a water-jacket thermostatic DW2/2 chamber combined with a “Clark-type” oxygen electrode and a computer-interfaced box CB1 (Hansatech Instruments, Kings Lynn, Norfolk, UK). The gametophytes were first detached from the substrate and resuspended several days prior to the experiment. Detaching gametophytes from their substrate does not affect their growth, their photosynthetic performance or their respiration (Fain & Murray, 1982). The gametophytes were held 12 h in the dark prior to the experiment, and then placed in the measurement chamber filled with 2 mL of PES and mixed with a magnetic stirrer. To prevent oxygen saturation, a quarter of the medium was renewed after each light step. To do so, a silk filtering mesh was used to retain the gametophytes in the media.

Respiration was measured in the dark, and gametophytes then underwent light-increasing steps of 20 min each (13 light steps, ranging from 2.5 to 500 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) using a halogen lamp (KL 2500 LCD, Schott, Germany) with a daylight cut-off filter (Schott, Germany). \(R_d\), NP and gross oxygen production (GP) rates were calculated based on fresh weight (FW, \(\mu\)mol O\(_2\) g\(_{\text{FW}}\) h\(^{-1}\)) and chlorophyll \(a\) content (chl \(a\), \(\mu\)mol O\(_2\) nmoles chl \(a\) \(^{-1}\) h\(^{-1}\)). FW was measured after collecting gametophytes on a silk filtering mesh that had previously been weighed.

**Fluorescence**
In vivo chl a fluorescence of the photosystem II (PSII) of gametophytes was measured using an underwater fluorometer (diving PAM; Heinz Walz, Effeltrich, Germany). The optimal quantum yield \((F_v/F_m)\) of PSII (Genty et al., 1989), a measure of the maximum efficiency of PSII, was measured using a 0.8 s saturating pulse (2500 µmol photons m\(^{-2}\) s\(^{-1}\)) of white light. We calculated the relative \(F_v/F_m\) ratio (rel. \(F_v/F_m\)) by dividing all data by the initial value measured at the beginning of the experiment in the dark.

The effective quantum yield of PSII \((\phi_{PSII})\), the efficiency of PSII photochemistry, was measured using a custom-made clip for Petri dishes to ensure a constant distance of 5 mm between the probe and the sample. The \(\phi_{PSII}\) was calculated according to Genty et al. (1989) and used to estimate the linear electron transport rate (relative electron transport rate, \(rETR\)) (Gevaert et al., 2003), an estimator of photosynthesis.

Non-photochemical quenching \((NPQ)\) indicates thermal dissipation of excess light in the PSII antennae, a photoprotective mechanism. We assumed that a stable \(NPQ\) level is reached after a 10-min illumination period, as shown in several microalgal species (Casper-Lindley & Björkman, 1998). \(F_m'\) values measured under very weak irradiance were higher than \(F_m\) values measured after dark-adaptation; therefore \(NPQ\) values were computed using the higher \(F_m'\) value instead of \(F_m\) (Serodio et al., 2005).

\(rETR\) and \(NPQ\) were measured on gametophytes at the end of each light-increasing step of 10 min each (12 light steps, ranging from 2.5 to 250 µmol photons m\(^{-2}\) s\(^{-1}\)). The \(NPQ\) value measured under the maximal irradiance is referred to as \(NPQ_{250}\).

\(P-I\) curves, \(rETR-I\)

The light-saturated maximum rate of GP \((P_{max})\), the light-saturated maximum rate of relative electron transfer \((rETR_{max})\), the light-limited initial slope \((\alpha)\), and the saturation
onset irradiance level \( (I_o) \) were calculated by plotting computed oxygen production rates and \( rETR \) against irradiance. \( P_{max} \) represents the maximal oxygen production, including all photosynthetic processes, while \( rETR_{max} \) is an estimation of the linear electron transfer in PSII, an indication of the overall photosynthetic capacity. Data were fitted using the model of Eilers & Peeters (1988) to each replicate with a least-square regression, using the Simplex method in the Statistica computer package (Statsoft, Tulsa, OK, USA).

Response to high irradiance

To study high light stress, the settled gametophytes in a Petri dish filled with PES at cultivation temperature were exposed to 500 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) for 2 h, and then, to dim light (10 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) to allow recovery. \( F_v/F_m \), \( \phi_{PSII} \), \( rETR \) and \( NPQ \) were measured every 30 min. Samples for pigment analyses were simultaneously collected at the start of the experiment, at the end of the light stress, and 2 h after the return to dim light. Gametophytes were detached from the substrate and the gametophyte suspension was filtered on a 20 \( \mu \text{m} \) silk filtering mesh and subsequently deep-frozen in liquid nitrogen for further pigment analyses.

Response to temperature

First, \( rETR-I \) curves were constructed for gametophytes acclimated 4 h at four different temperatures (5°C, 10°C, 15°C and 20°C) controlled by a thermo fluid circulator bath. During acclimation, \( F_v/F_m \) was measured hourly. Then, gametophytes underwent a progressive increase and decrease temperatures (2°C every 15 min) with an initial temperature set at 10°C. \( F_v/F_m \) was measured at the end of each temperature step.

Pigment analysis
Pigment contents of gametophytes were extracted by sonication and grinding in a cold mortar with methanol and methylene chloride. Extracts were centrifuged and supernatants were collected and dry-evaporated under nitrogen. Salt contents of the extracts were removed and the organic phase was evaporated and dissolved in methanol for injection. Pigment analysis was performed by high performance liquid chromatography (HPLC) (Beckman, system Gold, 126) with a reverse-phase column (C18 Allure, Restek). Separation was performed with a solvent delivery profile adapted from Arsalane et al., (1994). Pigment contents were quantified using specific absorption coefficients and normalised to the total pigment content. The conversion of violaxanthin into antheraxanthin and zeaxanthin was estimated by calculating the de-epoxidation ratio (DR):

$$DR = \frac{\text{antheraxanthin} + \text{zeaxanthin}}{\text{violaxanthin} + \text{antheraxanthin} + \text{zeaxanthin}}$$

Total chl $a$ concentrations were normalised to the FW of samples. Fucoxanthin and chl $c$ pigment concentrations normalised to chl $a$ (moles per 100 mol of chl $a$) were pooled, and referred to as the antenna pigment pool. Violaxanthin, antheraxanthin and zeaxanthin concentrations normalised to chl $a$ were pooled, referred to as the xanthophyll cycle pool.

**Statistical analyses**

Student’s $t$-test ($t$) (with Welch correction) was used to test for the difference between means of the photosynthetic parameters ($P_{\text{max}}$, $rETR_{\text{max}}$, $\alpha$ et $I_{d}$), the $F_{v}/F_{m}$, $NPQ$ values and pigment contents after the dataset was tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Response to light or temperature was fitted and response parameters were compared using Student’s $t$-test. The DR response was compared
within sites using a t-test for paired samples (t\(_p\)) after checking the data for normality. Adjustment of P values for multiple testing was done using the Holm method. The effect of temperature on the response parameters of light-response curves was analysed using a PERMANOVA (Anderson, 2001) in the vegan package of R software. Post hoc power analysis (1-β) were performed on non significant results using G*Power software which automatically determine the effect size (Faul et al., 2007). All experimental units had three replicates (n = 3).

Results

Comparison of the photosynthetic activity between the two sites

Gametophytes from the two sites showed differences in their photosynthetic parameters (Fig. 1). When normalized to chl \(a\), \(P_{\text{max(chla)}}\) values, the light-saturated maximal gross oxygen production values, were higher in Roscoff than in Wissant (\(t = 8.0\), Holm-adjusted \(P = 0.003\)), with 0.27 ± 0.01 \(\mu\)mol O\(_2\) nmol chl \(a^{-1}\) h\(^{-1}\) in Wissant, and 0.95 ± 0.15 \(\mu\)mol O\(_2\) nmol chl \(a^{-1}\) h\(^{-1}\) in Roscoff (Fig. 1 C). \(rETR_{\text{max}}\) values (22.74 ± 5.37 \(\mu\)mol e\(^{-}\) m\(^{-2}\) s\(^{-1}\) in Wissant and 15.90 ± 0.77 \(\mu\)mol e\(^{-}\) m\(^{-2}\) s\(^{-1}\) in Roscoff), the light-saturated maximal electron transfer rates, and \(P_{\text{max(FW)}}\) on a FW basis (56.47 ± 13.18 \(\mu\)mol O\(_2\) g\(_{FW}\)^{-1} h\(^{-1}\) in Wissant and 38.33 ± 1.12 \(\mu\)mol O\(_2\) g\(_{FW}\)^{-1} h\(^{-1}\) in Roscoff),were not significantly different between two sites (\(t = 2.9\), Holm-adjusted \(P = 0.133\) for \(rETR_{\text{max}}\); \(t = 2.8\), Holm-adjusted \(P = 0.142\) for \(P_{\text{max(FW)}}\) values) (Fig. 1 A-B), likely due to low statistical power resulting from small sample sizes (0.39 for \(rETR_{\text{max}}\) and 0.32 for \(P_{\text{max(FW)}}\)).
The same pattern appeared for the ascending slope under light limitation ($\alpha$ values).

$\alpha_{(chl_a)}$ values were significantly higher in Roscoff than in Wissant (respectively 0.005 ± 0.001 in Wissant and 0.023 ± 0.002 in Roscoff) ($t = 13.9$, Holm-adjusted $P = 0.001$) when expressed on a chl $a$ basis, whereas no differences were observed when expressed in electron transport rates or when expressed on a FW basis (respectively 0.37 ± 0.06 in Wissant and 0.40 ± 0.03 in Roscoff; $t = 0.68$, Holm-adjusted $P = 0.534$ for $\alpha_{(rETR)}$ and 1.15 ± 0.17 in Wissant and 0.99 ± 0.09 in Roscoff $t = 1.39$, Holm-adjusted $P = 0.305$ for $\alpha_{(FW)}$) which can also result from very low statistical power (respectively 0.09 and 0.19).

Considering the light saturation parameter ($I_k$), no significant differences were found between the two sites but the low statistical powers suggest that there were not enough replication to resolve the differences ($t = 1.95$, Holm-adjusted $P = 0.245$ and $1-\beta = 0.30$ for $I_k(rETR)$, $t = 1.77$, Holm-adjusted $P = 0.152$ and $1-\beta = 0.28$ for $I_k(FW)$ and $t = 1.13$, Holm-adjusted $P = 0.322$ and $1-\beta = 0.14$ for $I_k(chl_a)$). Despite this lack of significant differences, average $I_k$ values in gametophytes from Wissant were always greater than $I_k$ values in gametophytes from Roscoff (62.67 ± 20.20 µmol photons m$^{-2}$ s$^{-1}$ in Wissant, and 39.71 ± 1.73 µmol photons m$^{-2}$ s$^{-1}$ in Roscoff on an electron rate basis; 49.44 ± 9.83 µmol photons m$^{-2}$ s$^{-1}$ in Wissant, and 38.83 ± 3.42 µmol photons m$^{-2}$ s$^{-1}$ in Roscoff on a FW basis and 52.39 ± 13.49 in Wissant and 41.98 ± 8.56 in Roscoff on a chl $a$ basis).

Despite high irradiance (500 µmol photons m$^{-2}$ s$^{-1}$) in comparison with the culture conditions, there was no decrease in oxygen production observed.

Comparison of pigment contents
Pigment contents were similar at both sites (Table 2) except for chl c and violaxanthin contents, which were higher in Wissant than in Roscoff \((t = 4.69, \text{Holm-adjusted } P = 0.047\) for chl c and \(t = 4.26, \text{Holm-adjusted } P = 0.047\) for violaxanthin). When expressed on a total pigment content basis, no differences were observed in chl a contents in both sites, but chl a contents were slightly lower in Wissant and statistical power was low \((0.33)\). Therefore higher \(\Sigma XC:chl a\) pigment ratio may have been expected in Wissant, but the lack of significant difference is believed to be the result of a low statistical power \((0.53)\).

When chl a contents were expressed per unit fresh weight \((\text{respectively } 47.74 \pm 6.63 \text{nmoles } g_{\text{FW}}^{-1}\text{ in Roscoff and } 155.95 \pm 38.22 \text{nmoles } g_{\text{FW}}^{-1}\text{ in Wissant})\), they were significantly higher in Wissant \((t = 4.83, \text{Holm-adjusted } P = 0.036)\).

Comparison of photoprotective capacities

\(NPQ\) was measured along with \(rETR\) (Fig. 2). \(NPQ\) values after prolonged darkness \((12\text{ h, corresponding to the end of the dark period of culture conditions})\) were higher than those under weak irradiances. Maximal \(F_{m'}\) values were reached under an average irradiance of 20 \(\mu\text{mol photons m}^{-2}\text{ s}^{-1}\) in Roscoff, and 50 \(\mu\text{mol photons m}^{-2}\text{ s}^{-1}\) in Wissant.

\(NPQ\) progressively developed with increasing irradiance. Maximal \(NPQ\) values \((NPQ_{250})\) were reached at the highest applied irradiance \((250 \mu\text{mol photons m}^{-2}\text{ s}^{-1})\) and these values were significantly higher in gametophytes from Roscoff than in gametophytes from Wissant \((t = 3.5, \text{Holm-adjusted } P = 0.047)\), with \(NPQ_{250}\) values of 3.80 ± 0.42 in Roscoff and of 2.37 ± 0.56 in Wissant.
Intrinsic efficiency of PSII was altered in both populations, as indicated by the strong decline of rel. $F_{v}/F_{m}$ values (Fig. 3 A) when gametophytes were exposed to 500 µmol photons m$^{-2}$ s$^{-1}$ for 2 h. It reached a constant level, decreasing by 70% of the initial $F_{v}/F_{m}$ value after 2 h in both populations. In dim light, $F_{v}/F_{m}$ recovered at a level within 5% of the initial $F_{v}/F_{m}$ value after 1.5 h in Roscoff and after 4 h in Wissant.

During light stress, there was an increase in DR in gametophytes from Roscoff and from Wissant, corresponding to the progressive de-epoxidation of violaxanthin into antheraxanthin and zeaxanthin ($t_{(p)} = 7.9$, Holm-adjusted $P = 0.025$ for gametophytes from Wissant and $t_{(p)} = 8.9$, Holm-adjusted $P = 0.025$ for gametophytes from Roscoff) (Fig. 3 B). The increase in intrinsic efficiency of PSII under dim light was accompanied by a decrease in the DR values in gametophytes from Roscoff ($t_{(p)} = 11.6$, Holm-adjusted $P = 0.022$), indicating the reconversion of zeaxanthin and antheraxanthin into violaxanthin. In spite of the difference in the absolute values of DR measured at both sites after 2 h of strong illumination, the percent increase in DR was similar at both sites with an increase of 160% of the initial DR values. There was no significant decrease in DR values in Wissant after 2 h under dim light.

Comparison of the response to temperature

Increasing the temperature resulted in a decrease in $\alpha$ values in Wissant (Fig. 4 A), and an increase in $\text{rETR}_{\text{max}}$ and $I_k$ values for both sites (respectively Fig. 4 B and 4 C) (Table 3).

$NPQ_{250}$ was calculated at the end of each $rETR-E$ curves (Fig. 4 D). When exposed to 20°C, there was a slight decrease in the $NPQ_{250}$ values, in comparison with the values reached at 10°C.
The sensitivity of $F_v/F_m$ to temperature was tested on a broad range of temperature (Fig. 5). At 10°C, $F_v/F_m$ values in the dark were 0.52 ± 0.09 in Roscoff and 0.53 ± 0.06 in Wissant. With the progressive increase in temperature (from 10°C to 32°C), the rel. $F_v/F_m$ declined for temperatures greater than 20°C. There were no differences between sites in the response parameters from the regression analysis.

**Discussion**

Microscopic stages are thought to be the hardiest life-cycle stages of kelp (tom Dieck 1993). They form a seed bank which persists through stressful environmental conditions and ensures the persistence of species when unfavourable conditions occur, as it may happen during unusual heat waves (Ladah & Zertuche-Gonzales, 2007, Bartsch et al., 2013). Therefore, a great tolerance of *L. digitata* gametophytes to stressful environmental conditions is essential. In this study, we tested their physiological tolerance to irradiation and temperature stress in two populations with different environmental conditions along the French coast. The main result of the present study is that gametophytes of *L. digitata* were highly resistant to the irradiation and temperature treatments that might be locally encountered in the field. It is reinforced by the fact that sori were sampled during months when irradiation and temperature stress are not prevalent: their temperature and irradiation resistance may be even higher during summer.

We set out to study the incidence of high light stress on the photosynthesis of *L. digitata* gametophytes. Our results demonstrate that gametophytes can cope with prolonged high
irradiance stress, confirming the great potential for high light tolerance in kelp gametophytes (Iizumi & Sakanishi, 1994; Hanelt et al., 1997; Altamirano et al., 2004). This tolerance arises from the efficient thermal dissipation of excess light ($NPQ$) and, mainly, through the formation of antheraxanthin and zeaxanthin, which induce the conformational change in the light harvesting complexes (LHC) II (Jahns & Holzwarth, 2009). This zeaxanthin-dependent quenching is a slow component of $NPQ$, and Garcia-Mendoza & Colombo-Pallota (2007) have shown that kelp may lack the fast energy or pH-dependent quenching. However, the large xanthophyll cycle pigment pool may accelerate $NPQ$ development and thus compensate for this lack. Under dim light, epoxidation of zeaxanthin and antheraxanthin was virtually complete in less than 1 h. The slowly relaxing component of photoinhibition generally corresponds to the progressive re-activation of PSII or possibly to a conformational change of the LHC, and probably the aggregation if LHC due to zeaxanthin binding (Garcia-Mendoza & Colombo-Pallotta, 2007). Therefore, our study confirms that this efficient reversible conversion between violaxanthin, antheraxanthin and zeaxanthin in gametophytes (previously suggested by Hanelt et al., 1997 and Altamirano et al., 2004) is a widespread mechanism of fast-dynamic acclimation to abiotic stress in macroalgae (Fernandez-Marin et al., 2011).

Another possible mechanism protecting *L. digitata* gametophytes from rapid light fluctuations is the maintenance of $NPQ$ in the dark, a mechanism previously observed in microphytobenthos (Serôdio et al., 2005) and in several phytoplankton species (Casper-Lindley & Björkman, 1998; Cruz et al., 2011). In *Pelvetia canaliculata*, the $NPQ$ of chl $a$ fluorescence in the dark depends on the activation of violaxanthin de-epoxidase (Fernandez-Marin et al., 2011), which is triggered by acidification of the lumen due to
metabolic activity in the dark (Cruz et al., 2011). This activation may thus sustain ATP synthase activity (Casper-Lindley & Björkman, 1998; Serôdio et al., 2005), and prevent the formation of oxygen radicals. NPQ in the dark may therefore represent a type of sustained photoprotection, maintaining a dissipative state through pre-formed zeaxanthin or antheraxanthin, as observed here.

Acclimation to higher irradiances than the culture irradiance used in this experiment and/or an intermittent light exposure may have also provided a higher tolerance to the two-hour light stress of 500 µmol photons m\(^{-2}\) s\(^{-1}\) (Han and Kain, 1996; Lavaud et al., 2002). Along the French coast of the English Channel, irradiance of 500 µmol photons m\(^{-2}\) s\(^{-1}\) and more can be recorded during spring and summer low spring tides (Gevaert et al., 2003; Delebecq et al., 2011; Delebecq et al., 2013), but actual incident irradiances are generally deeply attenuated by the sporophyte canopy (Gerard, 1984) which may hypothesized that gametophytes display a great tolerance to high light.

This study also highlighted that the photosynthetic performance of *L. digitata* gametophytes were not negatively affected by short-term fluctuations in water temperature as it may be locally encountered in the field during periods of low tide. Gametophyte PSII — essential for the whole photochemistry process (Havaux & Tardy, 1996; Roleda, 2009) — shows high resistance to the temperatures currently encountered in the field. Increased temperature promotes electron transfer via the PSII through an increase in thylakoid membrane fluidity (Havaux & Tardy 1996) and results in an increase in maximum relative electron transfer rates (\(rETR_{\text{max}}\)) and in the onset of saturation parameters (\(I_k\)), as observed here. An increase in gross oxygen production with temperature can therefore be expected up to an optimum (Sukenik et al., 1987),
since the relationship electron transport rate and gross oxygen production is robust, 
provided that the temperature changes are not extremes (Morris & Kromkamp, 2003). 
Above an optimal temperature (above 20°C in this study), a complex set of inactivation 
and denaturation occur in thylakoïd, accompanied by the generation of elevated levels 
of reactive oxygen species which reduces photosynthetic capacity (Sharkey, 2005; 
Allakhverdiev et al., 2008) and also the ability to cope with high light stress (Roleda, 
2009). Superoptimal temperature also promotes electron-consuming processes (such as 
cyclic electron transfer) and dark respiration (Fain & Murray, 1982; Sharkey, 2005; 
Henkel & Hofmann, 2008) which can result in negative net photosynthesis.

However, short-term temperature responses do not provide insight into long-term 
individual survival with regard to temperature: temperature optima for photosynthesis 
can be several degrees higher than temperature optima for survival and growth 
(Davison, 1991) as it is not possible to evaluate the extent of sublethal stress. 
Concerning L. digitata gametophytes, tom Diek (1993) showed that they were able to 
survive 8 weeks at 23°C, demonstrating that gametophytes are moderately resistant to 
temperature extremes that are rarely encountered across its geographic range. Moreover, 
there is seasonal acclimation of the temperature response of gametophytes (Lee & 
Brinkhuis, 1988) and temperature resistance of PSII may be even higher in 
gametophytes sampled in warmer months or acclimated to higher temperatures 
(Mohring et al., 2013).

The two sites from which gametophytes were obtained were known to display different 
seasonal range of abiotic conditions (Berx & Hughes, 2009; Delebecq et al 2013), and a
strong photoacclimation to local environmental conditions of sporophytes of *L. digitata*

from the two same sites was previously highlighted (Delebecq *et al.*, 2013). Here, we
found some differences in the physiological characteristics of gametophytes between the
two sites, however the small replication did not exhibit the full physiological diversity
of the populations investigated, and replication was clearly not sufficient to resolve the
differences as indicated by the very low statistical power of our analysis (never
exceeding 53%). Moreover, results in the present study are confounded by the different
environmental conditions at the time of sampling in the two sites (*Table 1*) that may
influence the physiological response of gametophyte even after one month cultivation
under lab conditions, depending on the time-scale of acclimation to new light and
temperature treatments. More information on seasonal changes that can occur in the
gametophytes from Roscoff and Wissant would be necessary for a more complete
picture of the gametophyte response in the English Channel taking into account the
variation of the whole set of past and present environmental conditions, and to test if the
photosynthetic characteristics measured in sporophytes may be conserved in
gametophytes.

The higher chl *a* concentrations and fucoxanthin contents in gametophytes from
Wissant may suggest a greater light-harvesting efficiency and a higher density of
reaction centres (Gerard, 1988), which generally result in higher photosynthetic rates.
Although maximal photosynthetic rates (*rETR*<sub>max</sub> and *P*<sub>maxFW</sub>) in Wissant were not
significantly different from those in Roscoff, it is believed to result of low statistical
power, as indicated by the differences observed in *P*<sub>maxchl a</sub>. Hence, *P*<sub>maxchl a</sub> was higher in
Roscoff than in Wissant, indicating less efficient light use per chl *a* molecule in the
gametophytes from Wissant. This difference may be due to the self-shading of the LHC,
i.e. the fact that light absorption do not increase despite a higher pigment concentration (Falkowski & Raven, 1997). Daily underwater irradiances were shown to be similar in both sites at the time of sampling (Delebecq et al., 2013). As thermal acclimation is known to induce change in photoacclimation status (Machalek et al., 1996), those differences in photosynthetic characteristics may be partly due to the lower sea surface temperature in Wissant at the time of sampling (Table 1), if initial temperature encountered in the field still influence gametophyte’s response to light, even after one month cultivation.

Then, the larger violaxanthin content in Wissant may compensate for the large antenna size (higher fucoxanthin contents) which is known to decrease the probability of encounters between violaxanthin and violaxanthin de-epoxidase and the speed of de-epoxidation under high light (Garcia-Mendoza & Colomba-Palotta, 2007). It may also be due to the faster changing light conditions imposed by higher light attenuation in Wissant. Violaxanthin is also involved in the response to other abiotic stressors (Havaux & Tardy, 1996; Fernandez-Marin et al., 2011). Thus, a larger pool of violaxanthin may help gametophytes from Wissant to cope with other abiotic stressors not tested in this study.

We found no differences in the temperature response of the two sites with respect to the resistance of PSII quantum efficiency and increasing temperature. We expected that the wider temperature range encountered in Wissant may have induced a difference in thermostability of PSII. The small difference in temperature range may have not been sufficient to produce detectable differences. Moreover, at the time of sampling,
temperature was lower in Wissant (5°C) than in Roscoff (12°C) and this difference may have influenced the photosynthetic response of gametophytes in our study (Lee & Brinkhuis, 1988; Mohring et al., 2013). Beside thermal conditions and sampling time, the low replication size of our experiments may have been not sufficient to draw a well supported conclusion on the lack of difference between the two sites.

Regarding the temperature response of gametophytes, Bolton & Lüning (1982) suggested that Laminaria species may show sufficient phenotypic plasticity to adjust to the temperature range along its distribution and is not composed of differentiated temperature-adapted ecotypes throughout its range. However, notwithstanding similar growth patterns, physiological differences in the response to temperature may nevertheless occur among populations of perennial brown seaweeds (Henkel & Hofmann, 2008; Staehr & Wernberg, 2009). In order to test for difference in temperature response of the L. digitata’s populations along the French coast, further experiments need to compare gametophytes from (1) the two sites at different times of the year during their respective sporulation periods (Mohring et al., 2013) and (2) sites with more contrasting temperature ranges (Staehr & Wernberg, 2009). More generally, investigating the joint effect of light and temperature requires a multifactorial experiment (Fredersdorf et al., 2009).

Gametophytes appeared to be resistant to the light and temperature conditions currently experienced in the field. Regarding vulnerability to environmental conditions, physiological studies on the interactive effects of multiple abiotic stressors are required to improve our understanding of the microscopic stages in kelp (Fredersdorf et al., 2009). For instance, desiccation tolerance (Contreras-Porcia et al., 2012), ultraviolet
radiation (Roleda et al., 2006) and burial in sediment deposition (Roleda et al., 2011) all affect microscopic stages. While the southern geographic range-limit of *L. digitata* is thought to be set by inhibition of reproduction, as it was observed by Bartsch *et al.* (2013) when temperature reaches 18°C, survival of microscopic stages remains essential to cope with unusual stressful conditions. There is also an important need to obtain more information on the persistence of these stages in the field. It could help in defining which environmental conditions are really encountered in the field by the different microscopic developmental stages. Despite the present study failed to report local variation in the physiological response of *L. digitata*, it remains essential to take into account local variation in predicting the impact of fast-changing conditions in coastal areas.

Acknowledgments

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Figures legends

Fig. 1 Photosynthesis-irradiance curves (P-I curves) of gametophytes in *L. digitata* from Wissant (□) and Roscoff (●) (n = 3), expressed as (A) relative electron transport rate (rETR, µmol e⁻ m⁻² s⁻¹), (B) gross oxygen production on a fresh weight basis (GP, µmol O₂ gFW⁻¹ h⁻¹) and (C) gross oxygen production per unit chl a (GP, µmol O₂ nmoles chl a⁻¹ h⁻¹).

Fig. 2 NPQ of gametophytes in *L. digitata* from Wissant (□) and Roscoff (●) (n = 3) measured during the data collection for the rETR-I curves.

Fig. 3 (A) Relative optimal quantum yield (rel. *Fᵥ/Fₘ*) and (B) de-epoxidation ratio of violaxanthin into antheraxanthin and zeaxanthin of gametophytes in *L. digitata* from Wissant (□) and Roscoff (●) (n = 3). Gametophytes were first exposed to high irradiance (500 µmol photons m⁻² s⁻¹) for 2 h and then to dim white light (10 µmol photons m⁻² s⁻¹).

Fig. 4 Photosynthetic parameters (*α* (A), *rETRₘₐₓ* (B) and *Iₖ* (C)) and maximal NPQ (*NPQ₂₅₀*) (D) of gametophytes in *L. digitata* from Wissant (□) and Roscoff (■) (n = 3). Gametophytes acclimated at 5°C, 10°C, 15°C and 20°C exposed to increasing irradiance at the temperature of acclimation. Photosynthetic parameters (*α*, *rETRₘₐₓ* and *Iₖ*) were calculated using the model of Eilers & Peeters (1988).

Fig. 5 Relative optimal quantum yield (rel. *Fᵥ/Fₘ*) of gametophytes in *L. digitata* from Wissant (□) and Roscoff (●) (n = 3). Gametophytes initially kept at 10°C were
separately exposed to increases and decreases in temperature (2°C steps at 15 min
intervals). Data were fitted using a non-linear regression analysis ($y = a * e^{(b*x/c)} + d$).
Table 1 Environmental conditions in the two sites at the time of collection and 1 month prior to sampling. Environmental data were provided by T. Carriou from the Station Biologique de Roscoff and the Somlit network.

<table>
<thead>
<tr>
<th></th>
<th>Roscoff</th>
<th>Wissant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time of collection</td>
<td>1 month prior</td>
</tr>
<tr>
<td>Daylenght (hh:mm)</td>
<td>09:13</td>
<td>10:51</td>
</tr>
<tr>
<td>Daily surface irradiance (mol photons. m(^{-2}). j(^{-1}))</td>
<td>4.13</td>
<td>11.12</td>
</tr>
<tr>
<td>Light attenuation of PAR (m(^{-1}))</td>
<td>0.21</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Sea Surface Temperature (°C)</td>
<td>12.8</td>
<td>13.4 ± 0.8</td>
</tr>
<tr>
<td>Salinity</td>
<td>35.2</td>
<td>35.2 ± 0.0</td>
</tr>
<tr>
<td>Dissolved NO(_3) (µmol. L(^{-1}))</td>
<td>8.00</td>
<td>6.83 ± 1.07</td>
</tr>
<tr>
<td>Dissolved PO(_4) (µmol. L(^{-1}))</td>
<td>0.49</td>
<td>0.45 ± 0.05</td>
</tr>
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</table>
Table 2 Pigment composition (moles per 100 moles of all pigments) and pigment ratios (x 100) of gametophytes in *L. digitata* from Roscoff and Wissant (n = 3). Significant results (Holm-adjusted *P* < 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th>Pigment concentrations</th>
<th>Roscoff</th>
<th>Wissant</th>
<th><em>t</em></th>
<th>Holm-adjusted <em>P</em></th>
<th>1-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a (chl a)</td>
<td>54.51 ± 2.64</td>
<td>50.05 ± 2.93</td>
<td>1.96</td>
<td>0.243</td>
<td>0.33</td>
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<tr>
<td>Chlorophyll c (chl c)</td>
<td>8.15 ± 0.65</td>
<td>3.10 ± 1.75</td>
<td>4.69</td>
<td>0.047</td>
<td></td>
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<tr>
<td>Fucoxanthin</td>
<td>30.41 ± 3.00</td>
<td>37.85 ± 3.81</td>
<td>2.66</td>
<td>0.169</td>
<td>0.52</td>
</tr>
<tr>
<td>Violaxanthin (Vx)</td>
<td>2.11 ± 0.49</td>
<td>3.54 ± 0.31</td>
<td>4.26</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>β carotene</td>
<td>0.61 ± 0.37</td>
<td>0.69 ± 0.33</td>
<td>0.27</td>
<td>0.802</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Pigment ratios

<table>
<thead>
<tr>
<th>Pigment ratios</th>
<th>Roscoff</th>
<th>Wissant</th>
<th><em>t</em></th>
<th>Holm-adjusted <em>P</em></th>
<th>1-β</th>
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</thead>
<tbody>
<tr>
<td>Antenna:chl a pigment ratio</td>
<td>77.13 ± 9.11</td>
<td>89.65 ± 10.56</td>
<td>1.55</td>
<td>0.195</td>
<td>0.23</td>
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<tr>
<td>ΣXC:chl a pigment ratio</td>
<td>5.52 ± 1.53</td>
<td>9.28 ± 1.88</td>
<td>2.68</td>
<td>0.165</td>
<td>0.53</td>
</tr>
<tr>
<td>ΣXC:antenna pigment ratio</td>
<td>7.17 ± 2.01</td>
<td>10.28 ± 0.89</td>
<td>2.45</td>
<td>0.165</td>
<td>0.46</td>
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</table>
Table 3 Results of multivariate PERMANOVA analysis to test for differences in temperature and sites. Data were normalised and dissimilarities calculated as Euclidian distances. *P*-values were calculated from 999 permutations of the residuals under the reduced model. Significant results (*P* < 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Mean squares</th>
<th>Pseudo-F</th>
<th><em>P</em> (perm)</th>
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<td>( \alpha )</td>
<td>Site</td>
<td>1</td>
<td>0.0052</td>
<td>1.5444</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>3</td>
<td>0.0307</td>
<td>9.0127</td>
</tr>
<tr>
<td></td>
<td>Site x Temperature</td>
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<td>0.0001</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
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<td>0.0034</td>
<td></td>
</tr>
<tr>
<td>( I_k )</td>
<td>Site</td>
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<td>745.0</td>
<td>2.742</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>3</td>
<td>20189.3</td>
<td>76.516</td>
</tr>
<tr>
<td></td>
<td>Site x Temperature</td>
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<td>423.8</td>
<td>1.560</td>
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<td></td>
<td>Residual</td>
<td>20</td>
<td>271.7</td>
<td></td>
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<tr>
<td>( rETR_{max} )</td>
<td>Site</td>
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<td>15.35</td>
<td>0.717</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>3</td>
<td>1122.24</td>
<td>52.420</td>
</tr>
<tr>
<td></td>
<td>Site x Temperature</td>
<td>1</td>
<td>22.51</td>
<td>1.051</td>
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<tr>
<td></td>
<td>Residual</td>
<td>20</td>
<td>21.41</td>
<td></td>
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<tr>
<td>( NPQ_{max} )</td>
<td>Site</td>
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<td>11.2083</td>
<td>11.21</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>3</td>
<td>12.0839</td>
<td>12.08</td>
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<td></td>
<td>Site x Temperature</td>
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<tr>
<td></td>
<td>Residual</td>
<td>20</td>
<td>0.5527</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 3

(A) Relative fluorescence/maximum fluorescence (Rel. F/Fₘₖ) over time (h) for different conditions.

(B) DR (a parameter related to photosynthesis) over time (h) for different conditions.
Fig. 4