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## Effects of thermal history on intra- and transgenerational plasticity in *Laminaria pallida*

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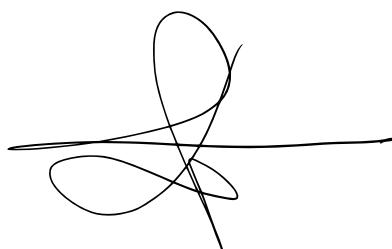
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'No data can be taken out of this work without prior approval of the thesis promoter / supervisor.'

To Anastasiya Laznya.

'I hereby confirm that I have independently composed this Master thesis and that no other than the indicated aid and sources have been used. This work has not been presented to any other examination board.'

Pierre Liboureau  
01/08/2021

A handwritten signature in black ink, appearing to read "Pierre Liboureau". It consists of a stylized, looped 'P' and 'L' followed by a more fluid, cursive section.

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## **Executive summary**

Climate change is one of the greatest challenges to sessile species worldwide. Kelps are crucially important as habitat-forming species in cold to temperate shallow ecosystems. As losses in abundance and range contractions are recorded for many species, research into acclimation and resilience of kelps to thermal stress is paramount. Phenotypic plasticity is the most important mechanism for sessile species to acclimate to rapidly emerging stressors and can be passed onto several generations. Here, we investigated the effects of long-term thermal stress on vegetative gametophytes of the kelp *Laminaria pallida* and the impact of thermal history on gametogenesis and thermal plasticity in microscopic F1 sporophytes. We hypothesized that a high thermal history would improve gametophyte reproduction as well as the tolerance of F1 sporophytes to heat stress. We also hypothesized that different genetic lines would yield different results, linked to female/maternal and inbreeding effects. We exposed vegetative gametophytes of *L. pallida* to 8°C and 20°C for 3.5 months. We assessed the effects of thermal history on the subsequent rate and success of gametophyte reproduction at 14°C (30 days), and on the survival, growth and photosynthetic efficiency of F1 sporophytes exposed to a range of temperatures (16 days). Our results showed a decrease in gametophyte fitness after long-term exposure to high temperatures. Gametophyte reproduction, however, was faster and more successful in high thermal history gametophytes, suggesting a link to reproductive seasonality in sporophyte reproduction and/or a potential effect of stress in inducing gametophyte reproduction. The F1 sporophytes with high thermal history had an increased photosynthetic efficiency but showed slower growth during early exposure to high temperatures. In contrast, once acclimated to thermal stress, growth rates were increased and similar independent of thermal history. Exposure to upper survival temperatures (23°C) resulted in decreased fitness and growth irrespective of thermal history. Overall, the effects of transgenerational plasticity on the response of F1 sporophytes to heat

stress were observed in the photosynthetic efficiency and, for some genetic lines, in the growth. Generally, significant genetic variation was observed in the effects of plasticity, linked to female/maternal effects. Intra- and transgenerational phenotypic plasticity is likely to improve the resilience of *Laminaria pallida* to climate change, but may favour some genetic lines, leading to loss of genetic diversity.

## Abstract

As climate change threatens marine ecosystems, sessile species such as kelps are heavily impacted by rapidly changing environmental conditions. In this context, phenotypic plasticity (genotype x environment interaction) is a key acclimation mechanism to improve resilience to high temperatures. Here, we investigated the effects of long-term thermal stress on intra- and transgenerational plasticity of the warm-temperate kelp *Laminaria pallida*. Our results highlight the effects of warm-temperature seasonality and heat stress in promoting gametophyte reproduction. Effects of parental thermal history on juvenile F1 sporophytes are more complex, with high thermal history sporophytes showing increased photosynthetic efficiency, but no definitive transgenerational effects on growth and survival. Significant genetic variation depending on female/maternal genetic lineage was observed for both intra- and transgenerational plasticity. Effects of gametophyte thermal history on reproduction and juvenile sporophyte growth differed based on female/maternal effects. Irrespective of thermal history, sporophyte fitness-related traits were optimal under mild to warm temperatures (14–20°C) but declined sharply when exposed to 23°C. *Laminaria pallida* therefore benefits from high temperature exposure during the haploid (gametophyte) life stage, but milder temperatures promote juvenile sporophyte growth and health.

## **Introduction**

Global warming is one of the greatest threats to ecosystems and biodiversity worldwide, with 86% of the oceans predicted to be impacted by 2050 (e.g., Henson et al., 2017; IPCC, 2019; Kröel-Dulay et al., 2015; Seddon et al., 2016). Species strategies to survive emerging environmental stressors include distribution shifts, acclimation (phenotypic plasticity) or genetic adaptations (Donelson et al., 2019). Sessile organisms are particularly vulnerable to climate change as natural distribution shifts and genetic adaptations may be too slow in the face of rapidly changing conditions (Atkins & Travis, 2010), posing a risk of range contraction and local extirpations. Consequently, phenotypic plasticity is critical in increasing the resilience of sessile species to climate change (Schlichting, 2003).

The true kelps, brown algae of the order Laminariales are key habitat-forming species in temperate to cold-water shores (Dayton, 1985; Mann, 1991). With some of the highest rates of primary production in marine ecosystems and three-dimensional structures, the ecological value of kelp forests ranges from nursery grounds and refuge from predators to food source (Hop et al., 2012; Roleda et al., 2007). By enhancing secondary production, they support complex, diverse ecosystems at all trophic levels (Oliver et al., 2018; Smale, 2020). Kelp forests also provide natural protection against coastal erosion and play an important role as significant long-term carbon sinks (Wiencke & Bischof, 2012). Additionally, Laminariales have a high economic value, being traditionally used as food and for alginate production, while more modern uses include medicine and cosmetic production and transformation into biofuels (e.g., Bartsch et al., 2008; Lorentsen et al., 2010; Wiencke & Bischof, 2012).

As sessile species, kelps are highly sensitive to rapid changes in environmental conditions. Many kelp populations are currently under threat due to ocean warming, with large-scale declines in kelp abundance and geographical range shifts being reported worldwide

(Krumhansl et al., 2016; Smale, 2020). In the Indian and Pacific Oceans, net decreases in abundance and biomass have been observed across several kelp genera (e.g., Johnson et al., 2011; Wernberg et al., 2016). North-Atlantic kelp populations are showing both southern edge contractions and northward shifts as waters warm (e.g., *Laminaria hyperborea*, *Laminaria ochroleuca*, *Saccharina latissima* and *Saccorhiza polyschides*: Casado-Amezúa et al., 2019; Teagle & Smale, 2018). Similarly, cold-water kelps are receding and being replaced by warm-water species in Japan (Kirihara et al., 2006).

Phenotypic plasticity is a key response to rapidly emerging environmental threats and describes phenotypic changes of individuals in response to new environmental conditions. The important role of phenotypic plasticity in increasing resilience to environmental stressors is shown in several studies (Charmantier et al., 2008; Nicotra et al., 2010; Seebacher et al., 2014). Plasticity is also thought to lead to genetic adaptations (e.g., Sommer, 2020), although evidence remains scarce (Merilä & Hendry, 2014).

Plasticity is divided in three major forms: acclimation, developmental and transgenerational. Short-term acclimation arises during stress but does not persist in the individual. Such acclimation is widely studied in kelps, which generally show strong resilience to heat stress until species-specific temperature thresholds (e.g., Burdett et al., 2019; Delebecq et al., 2016; Martins et al., 2017). Developmental plasticity can be retained by an individual, leading to improved responses upon further exposure to stressors (Byrne et al., 2020; Diaz et al., 2020; Palmer et al., 2012). This plasticity increases the resilience of individuals exposed to regular, cyclic stressors. In kelps, exposure to thermal stress led to lasting effects on growth, photosynthetic ability and reproduction (Martins et al., 2020; Roleda et al., 2007). Finally, transgenerational plasticity refers to the non-genetic transmission of phenotypic traits across generations. This improves the resilience of offspring during early life stages, which are often especially susceptible to stressors (e.g., Donelson et al., 2018; Marshall, 2008). Recent work

by Liesner et al. (2020) suggests the existence of thermal transgenerational plasticity in *Laminaria digitata*, although it also highlights the importance of cold seasons in its life cycle. Phenotypic plasticity allows species to survive emerging new stressors long enough for distribution shifts to happen or genetic adaptations to emerge (Herman & Sultan, 2011; Weigel & Colot, 2012). Developmental and transgenerational plasticities are often referred together as carry-over effects. They are generally driven by epigenetic changes, allowing rapid phenotypic adaptations as a response to specific environmental cues (Bell & Hellmann, 2019; Benson et al., 2020; Heard & Martienssen, 2014).

The ability for individuals to undergo developmental and transgenerational plasticity is both genetically variable and heritable (Liesner et al., 2020; Sultan et al., 2009; Vu et al., 2015). Maternal effects are the most common and have been studied in a variety of taxa (Galloway & Etterson, 2007; Marshall, 2008; Shama et al., 2014). Paternal effects also exist but are less widespread (e.g., Guillaume et al., 2016; Lacey, 1996; Latzel et al., 2014). Evidence of both maternal and paternal effects exist in Laminariales, although the former appears prevalent (Martins et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993; Zhang et al., 2007). Another genetic factor with significant impact on the life cycle of kelps is inbreeding. Indeed, the ability to self-fertilize has been observed in many kelp species (e.g., *Macrocystis pyrifera*: Carney et al., 2013; *Ecklonia cava*: Itou et al., 2019). The costs of inbreeding on the offspring are recorded to vary between species and populations. In *Macrocystis pyrifera*, costs are very high, with dramatic decreases in fitness and competitiveness of sporophytes (Camus et al., 2018; Raimondi et al., 2004). On the other hand, *Postelsia palmaeformis*, which forms small, scattered populations, showed few costs to inbreeding (Barner et al., 2011). Therefore, it is key to understand how different genetic effects influence phenotypic plasticity in kelps to better evaluate the potential of plasticity in mitigating the effects of climate change.

Priming is a commonly used agricultural technique, exposing parents and/or seeds to stressors to increase the resilience of offspring (e.g., Benson et al., 2020; Lämke & Bäurle, 2017). Such procedures have been widely used for decades to improve survival and yields and are already adapted to mitigate the effects of climate change on terrestrial crops (Mercé et al., 2020). A better understanding of kelp phenotypic plasticity will allow the establishment of priming techniques that can help to mitigate climate change effects as well as secure sustainable seaweed aquaculture and population restoration strategies. However, studies focusing on gametophyte priming and transgenerational plasticity are scarce and with contrasting results. Transgenerational effects were recently shown in two kelp species (*Laminaria digitata*: Liesner et al., 2020; *Ecklonia radiata*: Mabin et al., 2019). However, Jueterbock et al. (2021) highlighted the need for important developments in research before priming can be applied to kelps as it currently is to terrestrial crops.

Kelps are characterised by a heteromorphic life cycle that alternates between microscopic stages and macroscopic sporophytes. Mature diploid sporophytes release meiospores that differentiate into dioicous gametophytes. During gametogenesis, the female gametophytes form oogonia that produce non-motile eggs (Dayton, 1985). Additionally, oogonia release lamoxirene, a pheromone that triggers sperm release from antheridia on the male gametophyte which attracts sperm chemotactically (Maier et al., 2001). Following fertilisation, a diploid juvenile sporophyte is formed and closes the life cycle. Kelp reproduction is generally seasonal, leading different early life stages to be exposed to different environmental conditions. Development and transitions between life stages are triggered by environmental cues to maintain an optimised life cycle (Dayton, 1985). Therefore, changes in environmental conditions are likely to disrupt the life cycle and it is essential to study how transgenerational plasticity can be used to mitigate potential negative effects.

Juvenile sporophytes are especially susceptible to damage from environmental stressors and the important processes of gametophyte reproduction and recruitment success depends on optimum environmental conditions specific to each species (Martins et al., 2017; Roleda et al., 2007; tom Dieck (Bartsch) & de Oliveira, 1993). In turn, gametophytes have developed the ability to cope with unfavourable environmental conditions by slowing their metabolism and surviving extended periods of time in a vegetative state until conditions allow reproduction (Martins et al., 2017; Park et al., 2017). Prolonged vegetative phases facilitate long-term exposure of gametophytes to stressors to trigger phenotypic adaptations without disturbing the natural life cycle.

The split-fan kelp, *Laminaria pallida* (Greville), is mainly distributed on the South-West coast of Africa, between Danger Point in South Africa and Rocky Point in Namibia (Molloy, 1990; Rothman et al., 2015), but it has been also observed on some islands in the Southern Ocean (e.g., Ile Saint-Paul, Papenfuss et al., 1942). In South Africa, it is one of two dominant kelp species with *Ecklonia maxima*, while in Namibia it is the sole habitat-forming species. The distributional area of the species is characterized by strong upwelling and warm-temperate surface waters, from 11°C to 22°C (Demarcq et al., 2003). The largest populations are found south of Lüderitz, Namibia, with sea surface temperature maxima around 20°C. Northern populations, which are routinely exposed to higher temperatures, are generally sparser and more scattered (Nayar et al., 2018; Rothman et al., 2015). Sporophyte reproduction occurs year-round, although a strong peak is observed at the end of the Austral summer (i.e., end of March, Rothman et al., 2015). Gametophytes may be exposed to high temperatures during the autumn, but gametogenesis only begins when temperatures drop below 18°C (tom Dieck (Bartsch) & de Oliveira, 1993). Sporophyte recruitment typically happens during the winter, with juvenile sporophytes growing mostly between August – January (Dieckmann, 1978).

In South Africa, ecosystem services provided by kelp forests (*E. maxima* and *L. pallida*) were estimated at €130 million/year (Blamey & Bolton, 2018). Beach-cast *L. pallida* is traditionally collected for food consumption. Although widespread in South Africa, only 20% of the southern Namibian populations are harvested due to lack of access (Molloy, 1990). As exploitation of *L. pallida* biomass increases, proper management is required to preserve these fragile ecosystems while protecting the economy. Moreover, as seawater temperature is predicted to increase by 1-4°C (Meredith et al., 2019), *L. pallida* populations in northern Namibia risk being regularly exposed to temperatures near or above the upper sporophyte survival temperature of 23°C (tom Dieck (Bartsch) & de Oliveira, 1993), and southern populations will experience increasing thermal stress, potentially leading to habitat loss. Deeper knowledge of transgenerational effects in *L. pallida* will help to improve the management of existing populations and restoration initiatives in endangered locations.

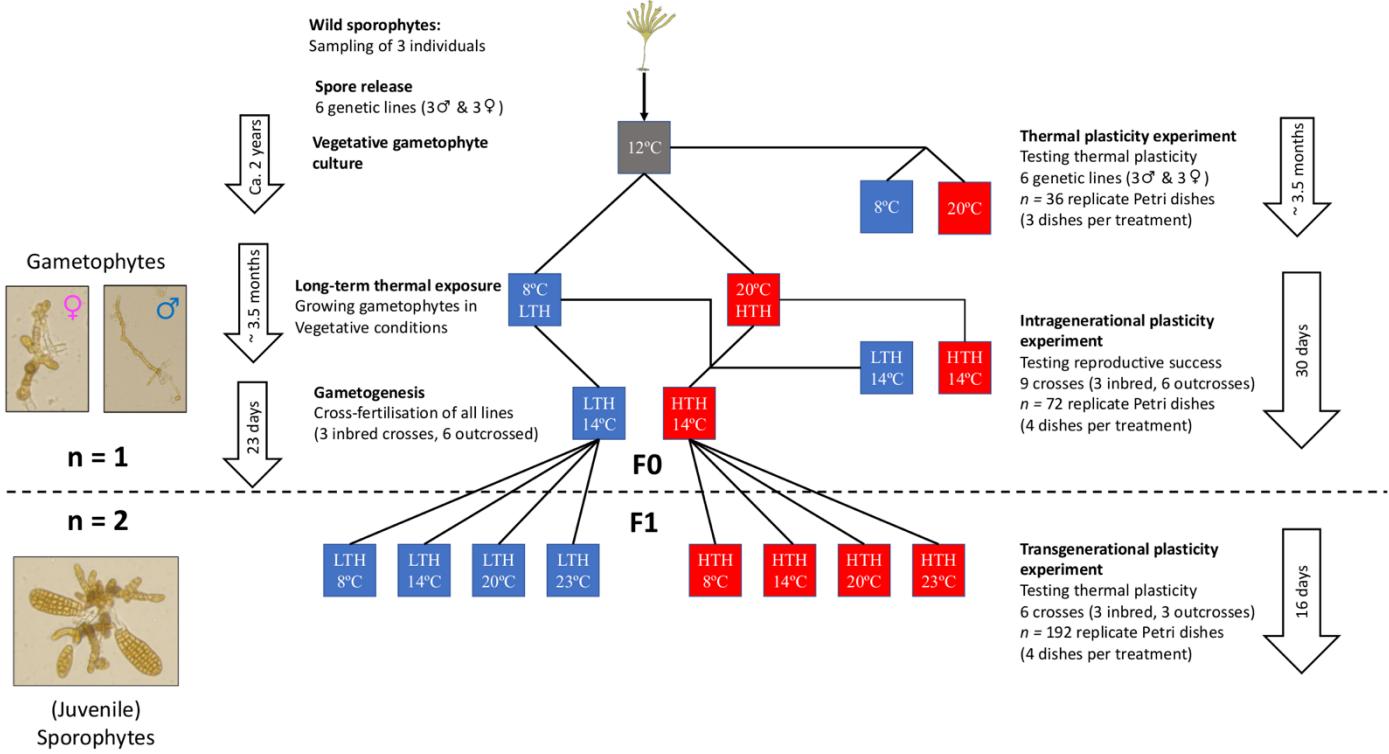
This study aims to investigate thermal plasticity of *Laminaria pallida* within and across early life stages. Gametophytes were exposed to thermal stress for ~3 months, similar to the vegetative duration in natural conditions, to evaluate whether thermal history of gametophytes leads to phenotypic plasticity in the speed and success of gametophyte reproduction. Finally, transgenerational effects were assessed by investigating whether the thermal tolerance of juvenile F1 sporophytes is affected by the thermal history of their gametophyte parents. We also investigated the potential differences in plasticity linked to genetic lineage.

We hypothesized that 1) a high thermal history would lead to increased gametophyte reproductive success. We further hypothesized that 2) offspring with a high parental thermal history would perform better at high temperatures (i.e., transgenerational carry-over effects). 3) We expected to observe a prevalence of female/maternal effects over male/paternal effects, as well as 4) decreased fitness and plasticity in inbred compared to outcrossed sporophytes.

## **Materials and methods**

### **1. Algal material**

Three mature sporophytes of *Laminaria pallida* were sampled from Swakopmund, Namibia (coordinates: -22.672, 14.522), near the Northern boundary of the species in July 2019. There, surface seawater temperature reaches 22°C during the Austral summer (Demarcq et al., 2003), close to the upper survival temperature (23°C) of *L. pallida* sporophytes (Martins et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993). Minima of 12°C (Demarcq et al., 2003) are recorded during the winter. Sori were cleaned and meiospores from each sporophyte were released separately in sterile seawater. After spore germination, male and female single-sex gametophyte stock cultures were established for each individual (numbered 1, 3 and 6) and maintained in a vegetative state in sterile half-strength Provasoli enriched seawater (PES; Provasoli, 1968) at 12°C under 3 µmol photons m<sup>-2</sup> s<sup>-1</sup> of red light and 16h:8h light:dark (L:D) cycle in a climate-controlled chamber (Fitoclima S600, Aralab, Lisbon, Portugal). Sterile artificial seawater (Tropic Marin Sea Salt, Wartenberg, Germany) with a salinity of 34 ± 1 ppm was used for maintenance and all experiments. The culture medium was changed monthly, until the beginning of the experiment (ca. 2y).



**Figure 1: Experimental design to test for thermal plasticity of gametophytes, intragenerational and transgenerational plasticity of *Laminaria pallida*.** Three fertile *L. pallida* sporophytes were sampled in the field (Swakopmund, Namibia). Meiospores were released and single-sex gametophytes were isolated and grown vegetatively for 2 years. All six cultures were exposed to 8°C and 20°C for 3.5 months. All genetic lines from both thermal histories were crossed at 14°C to produce nine crosses, of which three were inbred and six outcrossed (gametogenesis, 23–30 days). Following recruitment, six genetic lines were selected and transferred to four experimental temperatures (8, 14, 20, 23°C) to test for thermal plasticity (16 days). The dashed line shows the transition between haploid ( $n = 1$ ) and diploid ( $n = 2$ ) stages and. Between generations (F0, F1). HTH = High Thermal History, LTH = Low Thermal History.

## 2. Gametophyte exposure to long term temperature stress

Each single-sex culture of vegetative gametophytes was gently ground using a pestle and mortar, sieved and diluted to produce a stock solution of gametophyte fragments with lengths of  $\leq 100 \mu\text{m}$ . From each stock solution, the volume needed to achieve densities of  $\sim 500$  gametophytes  $\text{cm}^{-2}$  was added to Petri dishes (5.3 cm diameter, height 1.5 cm) containing 12 ml of half strength PES to measure the photosynthetic efficiency. Three replicate Petri dishes were used for each treatment (3 strains  $\times$  2 sexes  $\times$  2 temperatures  $\times$  3 replicates = 32 Petri dishes in total). The remaining volume of each strain suspension was poured evenly into two glass tubes filled with half strength PES. The gametophytes were allowed to recover from the mechanical stress induced by fragmentation for 14 days at 12°C, under  $3 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of red light in a 16h:8h L:D cycle. After this period, the fragmented gametophytes in the glass

tubes and Petri dishes were transferred to the experimental temperatures (8°C and 20°C) under the same light conditions. The gametophytes were maintained at both temperatures for ~3.5 months. The temperatures of 8°C and 20°C were chosen to reflect the annual mean minimum and maximum seawater temperature through the distribution range of *L. pallida* (Dieckmann, 1978). The culture medium was renewed weekly in the Petri dishes and every two weeks in the glass tubes. The gametophytes developing in the glass tubes were used in the following gametogenesis and sporophyte thermal tolerance experiments. Gametophyte fragmentation was performed to ensure new cells were developed under the experimental temperatures.

*2a. Photosynthetic efficiency:* The maximum photosynthetic yield ( $F_v/F_m$ ) was measured at the beginning and at the end of the long-term thermal exposure (~3.5 months) in the replicated Petri dishes.  $F_v/F_m$  was measured using a FluorPen FP 110 (PSI, Drásov, Czech Republic; Flash pulse: 20%, Super pulse: 70%, Actinic pulse: 10 $\mu$ mol) and used as a proxy for physiological performance. Gametophytes were dark acclimated for five minutes before the measurements. Two measurements were taken for each replicate, and the average  $F_v/F_m$  used.  $F_v/F_m$  data was normalised (divided by the mean values at day 0) to account for significant differences at the beginning between strains, allowing comparisons between strains.

### **3. Gametophyte reproduction at optimal temperature**

After ~3.5 months at 8°C and 20°C (thermal history), the gametophytes were transferred to 14°C by slowly increasing or decreasing the temperature at a rate of 3°C day<sup>-1</sup>. The gametophytes were allowed to acclimate to 14°C for 4 days. Gametophytes from each strain and each thermal history were then ground gently using a pestle and mortar, sieved and diluted in half strength PES to produce stock solutions of gametophytes fragments with

lengths  $\leq$  100 $\mu\text{m}$ . Densities from each strain stock were calculated. Crosses were obtained by combining one male and one female solution into Petri dishes (5.3 cm diameter, 1.5 cm height) containing 10 ml of half strength PES to achieve densities of  $\sim$ 600 gametophytes  $\text{cm}^{-2}$ . Within each thermal history (8°C and 20°C), each female gametophyte was crossed with all three males individually, resulting in a total of 9 crosses for each thermal history (Table 1). Three crosses were inbred, with male and female gametophytes coming from the same sporophyte, and six were outcrosses, with different parental sporophytes. Four replicate Petri dishes were used per treatment (9 crosses  $\times$  2 thermal histories  $\times$  4 replicates = 72 Petri dishes) to monitor gametophyte growth and reproduction. Five additional Petri dishes containing four cover slips each were prepared per cross and thermal history (9 crosses  $\times$  2 thermal histories  $\times$  5 replicates = 90 Petri dishes) to check for thermal tolerance differences in the microscopic sporophyte offspring.

**Table 1.** Summary of crosses used to evaluate gametophyte reproduction

Strain	♀ 1	♀ 3	♀ 6
♂ 1	Inbred	Outcrossed	Outcrossed
♂ 3	Outcrossed	Inbred	Outcrossed
♂ 6	Outcrossed	Outcrossed	Inbred

Post-fragmentation, gametophytes were allowed to settle and recover for 4 days at 14°C under 3  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{ s}^{-1}$  of red light in a 16h:8h L:D cycle. After this period the gametophytes were transferred to 17  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{ s}^{-1}$  of white light to induce gametogenesis in *L. pallida*. The 14°C temperature was chosen as it provides good gametogenic conditions (Martins et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993) while being at the midway point between thermal histories. The culture medium was changed every 11 days by the replacement of 7.5 ml of half strength PES per Petri dish.

*3a. Gametophyte growth:* Gametophyte area was measured on day 0 and day 6 of gametogenic conditions to quantify gametophyte growth before reproduction. For each

replicate, 12 randomly selected fields of view were photographed using a Nikon D90 camera (Nikon, Tokyo, Japan) mounted on a Zeiss Observer D1 inverted microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) at 100× magnification. The area of entire gametophytes present in each image was measured using ImageJ software (Schneider et al., 2012). The area measured excluded any eggs or sporophytes developed on female gametophytes. For each replicate, the average gametophyte area was calculated. Absolute growth rates (AGR) were calculated using the following formula:

$$AGR = \frac{(final\ area - initial\ area)}{experimental\ time\ (days)}$$

*3b. Gametogenesis:* The relative occurrence of different ontogenetic stages in female gametophytes was measured every 5 days for the first 20 days of gametogenic conditions and on day 28. In a minimum of 200 females per replicate, one of three ontogenetic stages: vegetative/oogonia, egg(s) released, and sporophyte(s) attached was assigned using a Zeiss Observer D1 inverted microscope. The most advanced developmental stage was assigned for each female gametophyte fragment. Sporophytes were considered as soon as the first cell division was visible in the zygote. For statistical analyses, gametogenesis rates were compared between crosses and thermal histories on day 10 since at least one cross showed over 80% of reproductive females (egg released or sporophyte attached). Reproductive success was evaluated as the percentage of female gametophytes with sporophytes after 28 days.

*3c. Sporophyte recruitment:* Recruitment capacity of sporophytes was evaluated as the absolute number of sporophytes per female gametophyte after 30 days. Sporophytes unattached to female gametophytes and with non-polar irregular morphology were not counted as they were considered resulting from parthenogenesis, therefore representing unsuccessful recruits (tom Dieck, 1992). The total sporophyte density was evaluated by

counting sporophytes in  $\geq$  60 fields of view (Zeiss Observer D1 inverted microscope; 100 $\times$  magnification) per replicate. Female gametophyte density was measured on day 28.

#### 4. Juvenile offspring sporophyte exposure to thermal stress

After 23 days in reproductive conditions, microscopic offspring sporophytes with a length of  $\sim$ 225  $\mu\text{m}$  developed in the different crosses and thermal histories. Offspring sporophytes from six crosses were randomly selected from the initial nine prepared, ensuring that each male and each female were represented in two crosses, and that three crosses were inbred and three were outcrossed (Table 2).

**Table 2.** Summary of crosses used to evaluate thermal plasticity of juvenile F1 sporophytes

Strain	♀ 1	♀ 3	♀ 6
♂ 1	Inbred	Outcrossed	-
♂ 3	-	Inbred	Outcrossed
♂ 6	Outcrossed	-	Inbred

From each Petri dish, each of the four cover slips containing microscopic sporophytes was transferred to a different target experimental temperature (8, 14, 20 and 23°C  $\pm$  0.5°C). Target temperatures were reached by slowly increasing or decreasing the temperature at a rate of 3°C day $^{-1}$ . Sporophytes were exposed to each target temperature for 16 days. Temperatures of 8, 14 and 20°C were chosen to represent the thermal range experienced in nature through the distributional range (Dieckmann, 1978, 1980) and 23°C was chosen as the upper survival temperature (Martins et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993) to easily check for response differences between thermal histories. Four large Petri dishes (8.9 cm diameter, height 2.5 cm) containing one cover slip each and 25 ml of half-strength PES were used for each treatment (6 crosses  $\times$  2 thermal histories  $\times$  4 experimental temperatures  $\times$  4 replicates = 192 Petri dishes). Experiments were conducted in temperature-controlled climatic chambers

(Fitoclima S600, Aralab, Lisbon, Portugal), with 17  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$  of white light in a 16h:8h L:D cycle.

*4a. Sporophyte density:* Sporophyte density was measured to assess the effect of experimental temperatures and thermal histories on the survival capacity of microscopic offspring sporophytes. Sporophyte densities were quantified at the beginning (day 0) and the end (day 16) of the thermal treatment. For each replicate, sporophytes were counted in a minimum of 50 fields of view (Zeiss Observer D1 inverted microscope; 100 $\times$  magnification). Data was normalised to account for discrepancies in initial sporophyte densities between crosses, thereby allowing comparisons between crosses.

*4b. Photosynthetic efficiency:* Photosynthetic efficiency was measured on days 0 and 16 to estimate the treatment effects on the physiological health of microscopic sporophytes. A FluorPen FP 110 (PSI, Drásov, Czech Republic) was used to measure the maximum photosynthetic yield ( $F_v/F_m$ ) as well as the response to a light curve (Light curve 1, Flash pulse: 20%, Super pulse: 40%, Actinic pulse: 18  $\mu\text{mol}$ ) for each replicate. The light curve response was used to calculate the relative maximum electron transport rate (rETRmax) using the Phytotools package in R software (R Core Team, 2021; Silsbe & Malkin, 2015). Sporophytes were dark acclimated for five minutes before the measurements. Data was normalised (divided by the mean values at day 0) to account for significant differences at the beginning between sporophytes from different crosses, allowing comparisons between crosses.

*4c: Sporophyte growth:* Sporophyte length was quantified at day 0 and after 8 and 16 days of thermal exposure. The length of 30 sporophytes was measured per replicate using ImageJ software (Schneider et al. 2012), corresponding to 20 randomly photographed fields of view, with a maximum of two sporophytes from each picture. A Nikon D90 camera mounted on a Zeiss Observer D1 inverted microscope (100 $\times$  magnification) was used for

measurements on day 0 and day 8, while on day 16 a Canon Powershot A640 camera mounted on a Zeiss Axiovert 40 (Zeiss, Oberkochen, Germany; 40 $\times$  magnification) was used due to the larger sporophyte sizes. The average sporophyte length was calculated for each replicate dish, and the AGR was estimated according to the formula used for gametophyte growth above.

## 5. Statistical Analysis

Data were analysed using SPSS 27 software (IBM corp., Armonk, NY, USA) and the PERMANOVA module of Primer 6 software (Anderson, 2001; McArdle & Anderson, 2001). Data was tested for normality within groups using the Shapiro-Wilk test and homoscedasticity using Levene's test in SPSS. The normalised  $F_v/F_m$  of gametophytes was analysed under a two-factor ANOVA (fixed factors: strain and temperature). Gametophyte absolute growth rate (square root transformed), percentage of reproductive females after 10 days, percentage of female gametophytes with sporophytes after 28 days and absolute number of sporophytes per female gametophyte data were also analysed under a two-factor ANOVA (fixed factors: cross and thermal history). Sporophyte density (log-transformed) and sporophyte length-AGR data were analysed under a three-factor ANOVA (fixed factors: cross, thermal history and experimental temperature). Post-hoc Tukey tests with Bonferroni corrections were conducted to determine differences between treatments when significant main effects or interactions were found. Differences between groups were considered significant when  $p < 0.05$ .

Sporophyte photosynthetic data did not fulfil the ANOVA assumptions of normality and homoscedasticity, and thus was analysed using PERMANOVA under a three-factor design (fixed factors: cross, thermal history and experimental temperature). Analyses were performed with Euclidean distances and 9999 permutations. Post-hoc pairwise t-tests

comparisons were performed to evaluate differences between treatments when significant main effect or interactions were found. Differences between groups were considered significant when  $p < 0.05$ .

## Results

### Gametophyte exposure to long term stress

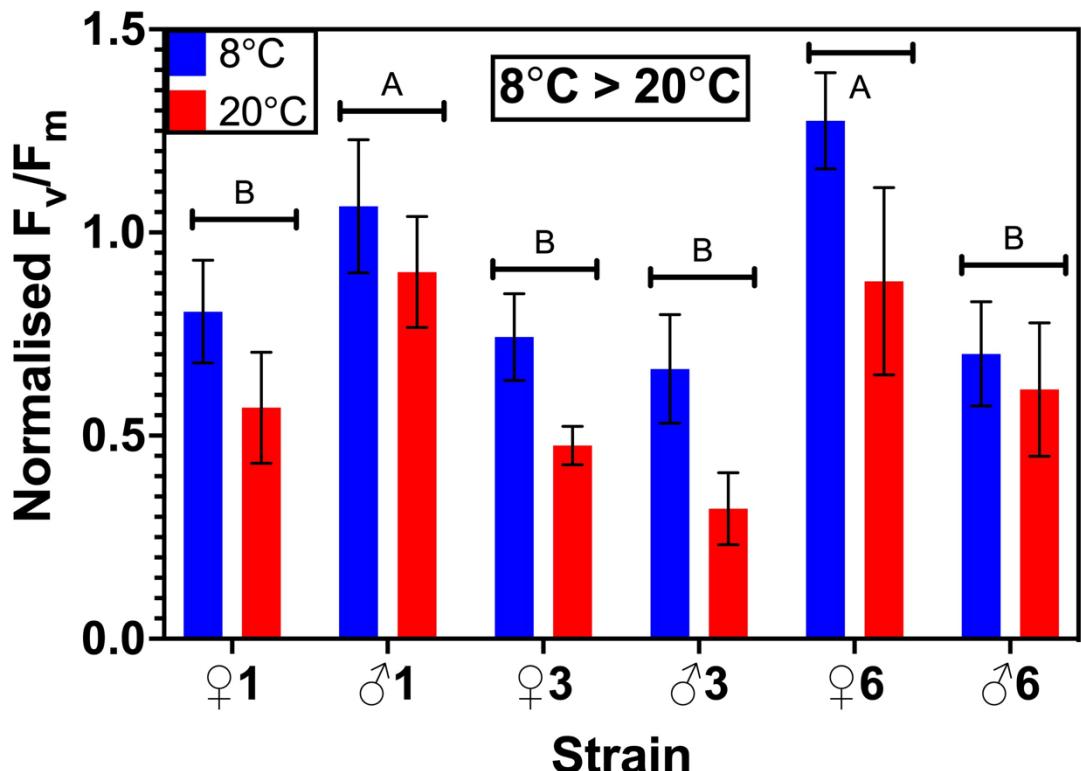
#### Photosynthetic efficiency

The normalised maximum quantum yield of PSII ( $F_v/F_m$ ) differed significantly with temperature and strain, but there were no interactions between the two factors (Table 3, Fig. 2). Normalised  $F_v/F_m$  was significantly higher (1.4-fold) in gametophytes exposed to low temperature (8°C) compared to high temperature (20°C), irrespective of the strain. The gametophyte strains  $\Omega$  1,  $\Omega$  3,  $\sigma$  3 and  $\sigma$  6 showed significantly lower (1.7-fold)  $F_v/F_m$  values compared to strains  $\sigma$  1 and  $\Omega$  6.

**Table 3.** ANOVA for the effects of strain and temperature on the  $F_v/F_m$  of *Laminaria pallida* gametophytes. The post-hoc results are presented in Fig. 2

Factor	df	SS	MS	F	P
Strain	5	1.56	0.31	16.33	<b>&lt;0.001</b>
Temperature	1	0.55	0.55	28.93	<b>&lt;0.001</b>
Strain × Temperature	5	0.10	0.02	1.01	0.434
Residual	24	0.460	0.019		

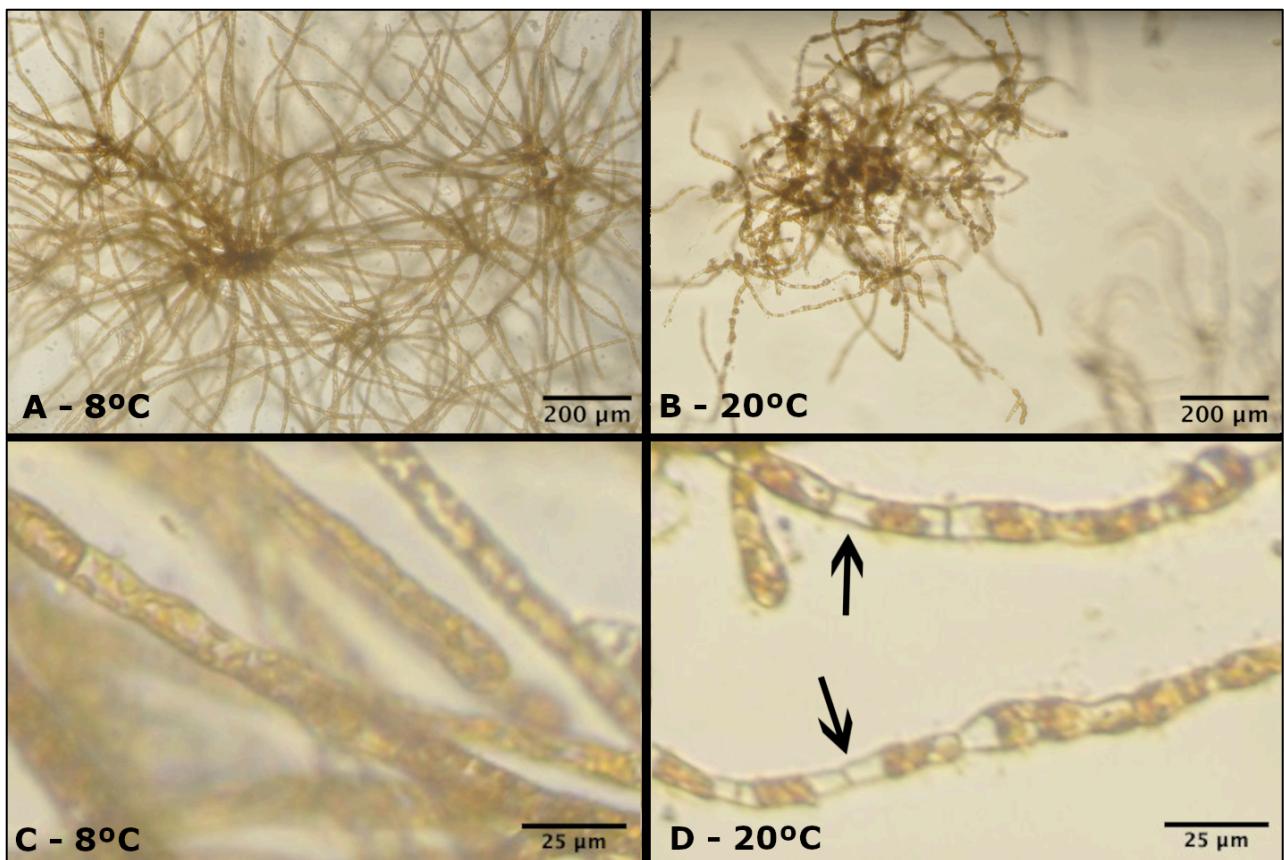
Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 2: Effect of long-term temperature stress ( $8^{\circ}\text{C}$  and  $20^{\circ}\text{C}$ ) on the maximum photosynthetic yield of PSII ( $F_v/F_m$ ) of different *Laminaria pallida* gametophyte strains.** Bar plots with mean and error bars with standard deviation ( $n = 4$ ). Different letters indicate differences between strains. See table 3 for statistics.

### Gametophyte health

After  $\sim 3.5$  months at  $8^{\circ}\text{C}$  and  $20^{\circ}\text{C}$ , large vegetative clusters of gametophytes developed, making it difficult to evaluate their density or growth increase, hence gametophyte fitness could only be evaluated qualitatively. Overall, the gametophyte clusters at  $8^{\circ}\text{C}$  were extended and formed large lattices (Fig. 3A), with uniformly brown cells (Fig. 3C). However, gametophytes exposed to  $20^{\circ}\text{C}$  formed denser, coiled up clusters (Fig. 3B), with many transparent cells, indicating high stress or death (Fig. 3D).



**Figure 3: Female gametophytes of *Laminaria pallida* after ~3.5 months of temperature exposure (8°C and 20°C). A, B. Gametophyte vegetative clusters. C, D. Gametophyte cells within the clusters. Arrows point to dead cells.**

### Gametophyte reproduction at optimal temperature

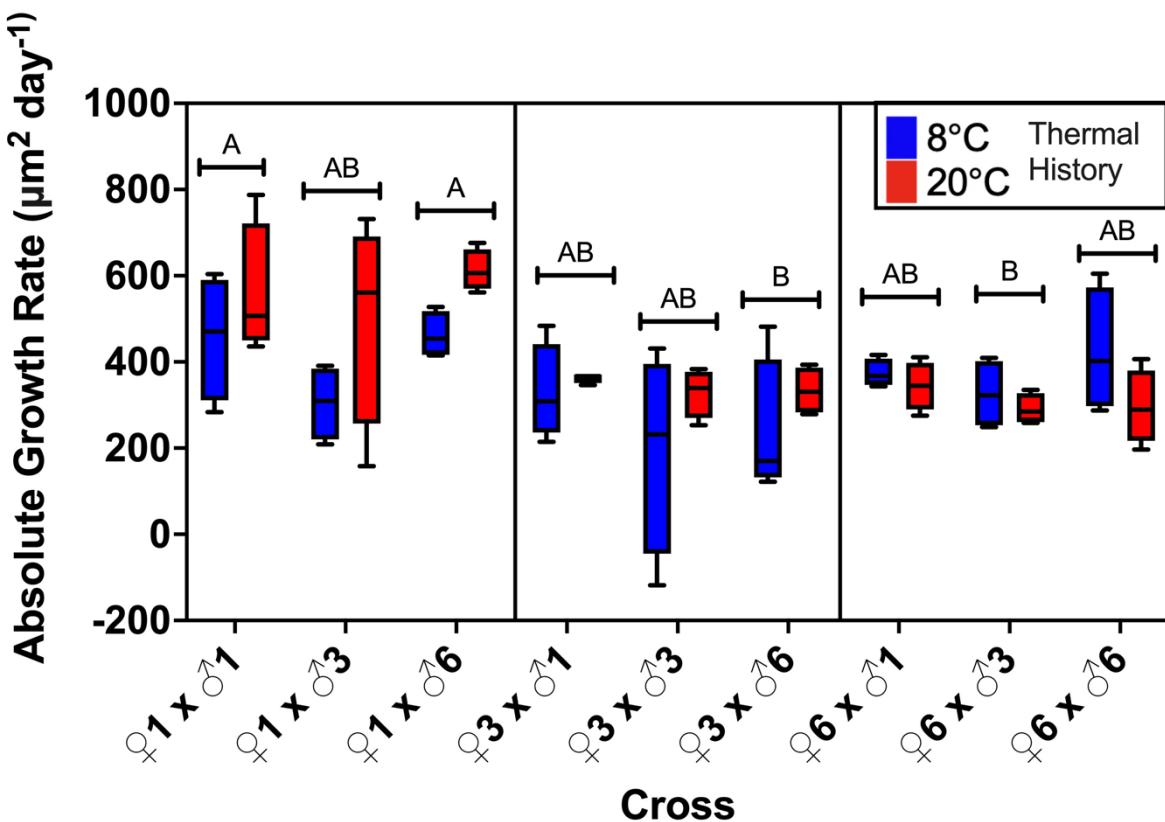
#### Gametophyte growth

Absolute growth rates (AGR) of gametophytes during the first 6 days of gametogenic conditions differed significantly only due to crosses (Table 4, Fig. 4). Two of the crosses with the female strain 1 ( $\text{♀} 1 \times \text{♂} 1$  and  $\text{♀} 1 \times \text{♂} 6$ ) had significantly higher (1.6-fold) growth rates than  $\text{♀} 3 \times \text{♂} 6$  and  $\text{♀} 6 \times \text{♂} 3$  crosses.

**Table 4.** ANOVA for the effects of cross and thermal history on the absolute growth rate for gametophyte area of *Laminaria pallida* after 6 days in gametogenic conditions. The post-hoc results are presented in Fig. 4.

Factor	df	SS	MS	F	P
Cross	8	309.2	38.63	4.66	<b>&lt;0.001</b>
Thermal history	1	24.43	24.43	2.95	0.092
Cross × Thermal history	8	99.78	12.47	1.51	0.177
Residual	53	438.93	8.28		

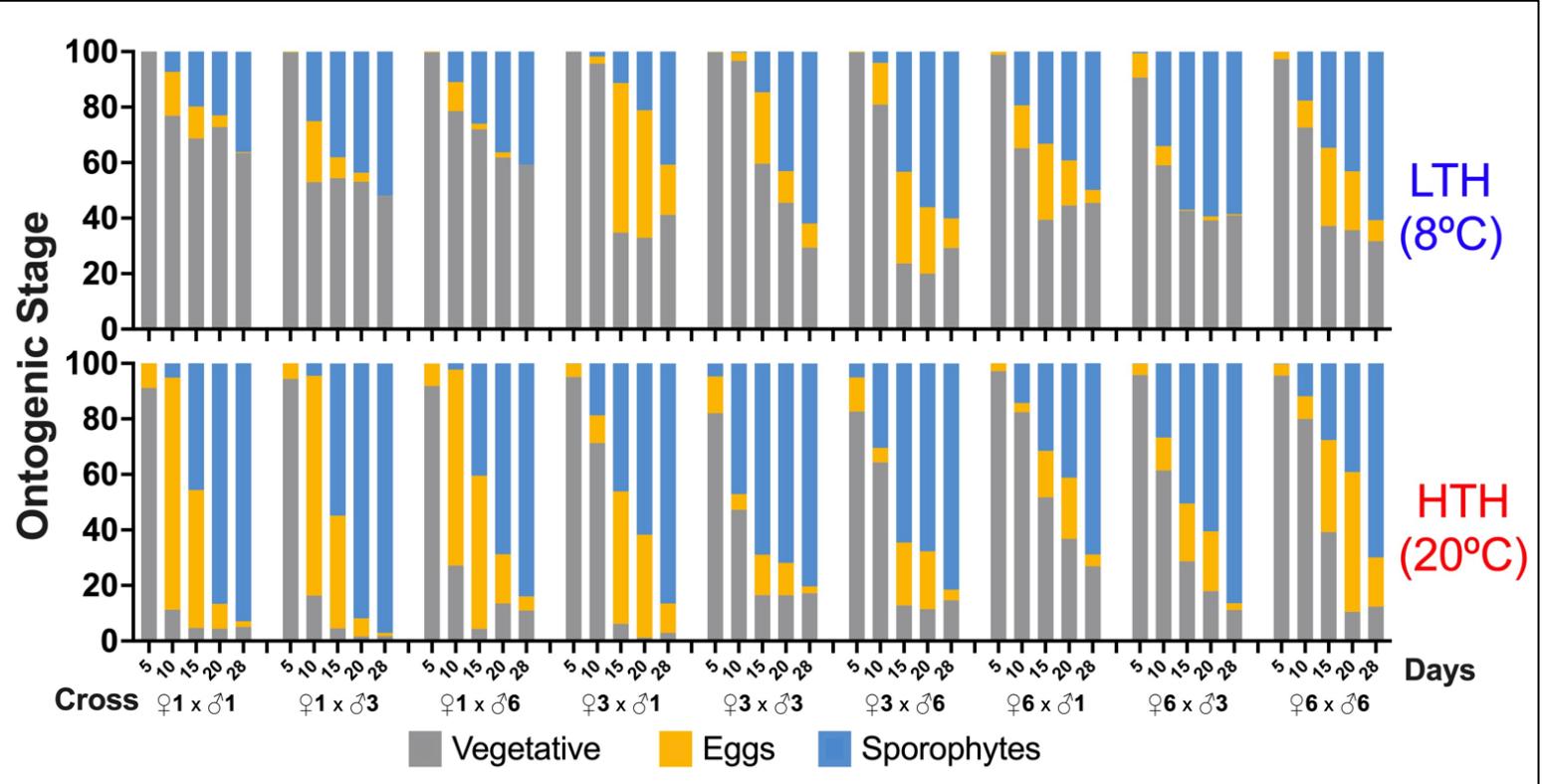
Significant interactions or main effects are highlighted in bold. Df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 4: Effect of thermal history on the absolute growth rate of gametophytes from different crosses after 6 days under gametogenic conditions.** Box plots with median, boxes for 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers indicating min and max values ( $n = 4$ ). Panels separate crosses with different females. Different letters indicate differences between crosses ( $p < 0.05$ ). See table 4 for statistics.

### Gametogenesis overview

Overall, gametogenesis was faster and more successful in gametophytes with high thermal history (Fig. 5). In most of the crosses, more than 80% of female gametophytes with high thermal history were reproductive with developed eggs and sporophytes after 15 days, whereas much lower percentages of female gametophytes with low thermal history became reproductive (30% - 70%). The same pattern was observed even after 28 days. For both thermal histories, the pattern of the temporal reproductive development was similar for crosses within the same female gametophyte strain but varied in crosses within the same male strain.



**Figure 5: Effect of thermal history on the female gametogenesis of different crosses over time (28 days).** 100% stacked column charts with means of each ontogenetic stage ( $n = 4$ ). LTH = Low Thermal History, HTH = High Thermal History.

The relative abundance of reproductive females (i.e., gametophytes with released eggs and/or attached sporophytes) after 10 days showed significant cross  $\times$  thermal history interactions (Table 5, Fig. 6A). Gametophytes with high thermal history (20°C, HTH) exhibited higher female reproductive success in all the crosses with ♀1 (3.8, 1.8 and 3.4-fold when crossed with ♂1, ♂3 and ♂6, respectively) and ♀3 (6.7, 15.5 and 1.9-fold when crossed with ♂1, ♂3 and ♂6, respectively) compared to low thermal history gametophytes (8°C, LTH). In contrast, LTH enhanced female reproductive success (2.0-fold) only in the cross ♀6  $\times$  ♂1 compared to a HTH. In the LTH gametophytes, the reproductive success pattern was similar in the crosses with ♀1 and ♀6; higher values were observed when both these females are crossed with ♂3 than when crossed with ♂1 and ♂6. The lowest reproductive success occurred in the crosses ♀3  $\times$  ♂1 and ♀3  $\times$  ♂3. In HTH gametophytes, female

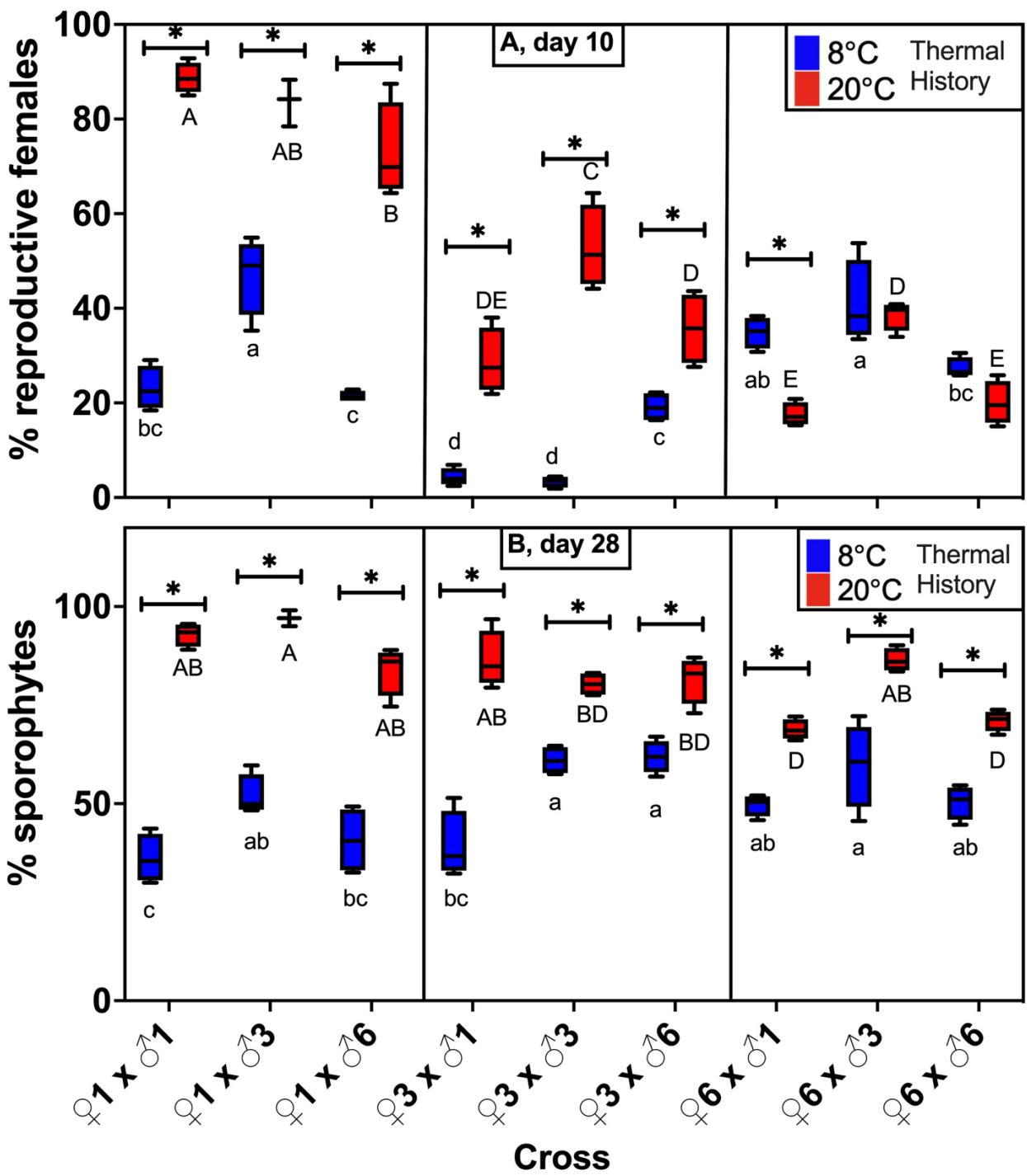
reproductive success was significantly greater in crosses with ♀1 compared with all the other crosses.

The relative abundance of females with sporophytes after 28 days showed significant cross × thermal history interactions (Table 5, Fig. 6B). For all crosses, HTH female gametophytes were significantly more reproductive than LTH ones. The largest differences were observed in crosses with ♀1 (2.6, 1.9 and 2.1-fold higher in crosses with ♂1, ♂3 and ♂6, respectively). In the HTH gametophytes, the relative sporophyte presence was higher in the cross ♀1 × ♂3 (97%) than in two of the crosses with ♀3 (♀3 × ♂3 and ♀3 × ♂6; mean value of 81%) and with ♀6 (♀6 × ♂1 and ♀6 × ♂6; mean value of 70%). On the other hand, the female gametophytes from the crosses ♀3 × ♂3, ♀3 × ♂6 and ♀6 × ♂3 with LTH developed a higher proportion of sporophytes (mean value of 61%) compared to the crosses ♀1 × ♂1, ♀1 × ♂6 and ♀3 × ♂1 (mean value of 39%).

**Table 5.** ANOVA for the effects of cross and thermal history on the relative abundance of reproductive female gametophytes of *Laminaria pallida* after 10 and 28 days in gametogenic conditions. The post-hoc results are presented in Fig. 6.

Factor	df	SS	MS	F	P
% Reproductive female gametophytes, day 10					
Cross	8	16292.94	2036.62	64.98	<0.001
Thermal History	1	10268.89	10268.89	327.62	<0.001
Cross × Thermal history	8	13415.898	1676.99	53.50	<0.001
Residual	53	1661.246	31.34		
% Female gametophytes with sporophytes, day 28					
Cross	8	2105.45	263.18	8.40	<0.001
Thermal history	1	19332.97	19332.97	617.04	<0.001
Cross × Thermal history	8	3488.85	436.11	13.92	<0.001
Residual	53	1669.59	31.33		

Significant interactions or main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 6: Effect of thermal history on the relative abundance of ontogenetic stages in female gametophytes (egg released or sporophyte formed) in different crosses in gametogenic conditions.** A: Relative abundance of reproductive females (egg released or sporophyte formed) after 10 days in gametogenic conditions. B: Relative abundance of females with sporophyte(s) formed after 28 days in gametogenic conditions. Box plots with median, boxes for 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers indicating min and max values (n = 4). Panels separate crosses with different females. \* indicates a significant difference between thermal histories within cross. For each thermal history, different letters indicate differences between crosses ( $p < 0.05$ , uppercase letters for 20°C thermal history and lowercase for 8°C thermal history). See table 5 for statistics.

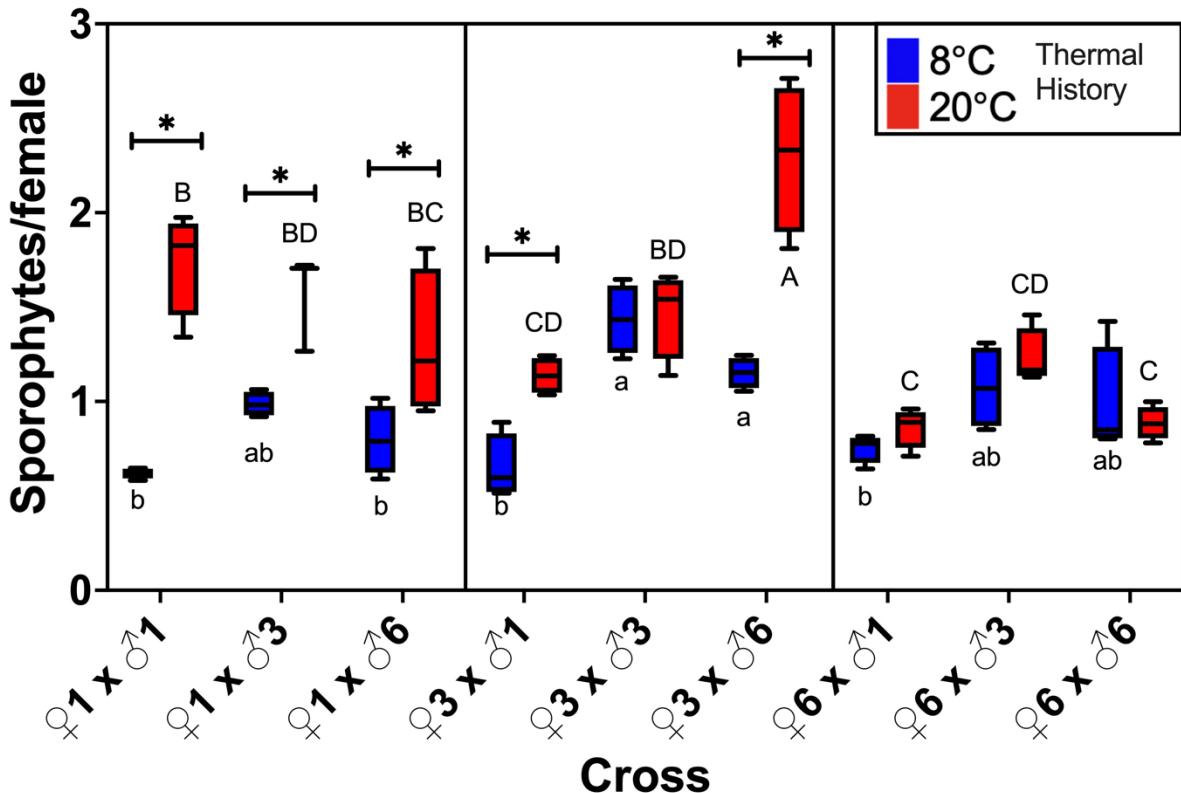
## Juvenile sporophyte recruitment

Sporophyte recruitment showed a significant cross  $\times$  thermal history interaction (Table 6, Fig. 7). HTH gametophytes produced significantly more sporophytes per female in all the three crosses involving ♀1 and in two of the crosses with ♀3 compared with LTH gametophytes. Conversely, there were no crosses in which LTH recruitment exceeded HTH recruitment. Gametophyte thermal history had no influence on sporophyte density in all the crosses with ♀6 and in the ♀3  $\times$  ♂3 cross. In LTH gametophytes, the numbers of sporophytes per female gametophyte were significantly greater (1.8-fold) in two of the crosses involving ♀3 (♀3  $\times$  ♂3 and ♀3  $\times$  ♂6) than in all the crosses with ♂1 and in the ♀1  $\times$  ♂6 cross. Recruitment success was higher (1.8-fold) in the cross ♀3  $\times$  ♂6 than in all the other crosses in the HTH gametophytes.

**Table 6.** ANOVA for the effects of cross and thermal history on the recruitment of sporophytes in gametophytes of *Laminaria pallida* after 30 days in gametogenic conditions. The post-hoc results are presented in Fig. 7.

Factor	df	SS	MS	F	P
Sporophytes/female gametophyte					
Cross	8	5.40	0.674	15.15	<b>&lt;0.001</b>
Thermal history	1	3.55	3.55	79.74	<b>&lt;0.001</b>
Cross $\times$ Thermal history	8	3.25	0.41	9.11	<b>&lt;0.001</b>
Residual	53	2.36	0.05		

Significant interactions or main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 7: Effect of thermal history on the absolute number of sporophytes per female gametophyte in different crosses after 30 days in gametogenic conditions.** Box plots with median, boxes for 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers indicating min and max values (n = 4). Panels separate crosses with different females. \* indicates a significant differences between thermal histories per cross. For each thermal history, different letters indicate differences between crosses ( $p < 0.05$ , uppercase letters for 20°C thermal history and lowercase for 8°C thermal history). See table 6 for statistics.

### Effects of parental thermal history on juvenile sporophytes

#### Sporophyte density

Overall, increases in sporophyte density were observed in the ♀1 crosses with LTH over the 16 days (normalised density  $> 1$ ), indicating ongoing reproduction of these gametophytes and thus the accumulated formation of sporophytes (Fig. 8). Similarly, increases in sporophyte density were also observed in the remaining crosses at 8, 14 and 20°C, regardless of the parental thermal history. At the higher temperature (23°C), further maturation seems to be prevented, and the stable sporophyte densities reflect survival rather than additional development in these crosses. In the two HTH ♀1 crosses, sporophyte densities remained

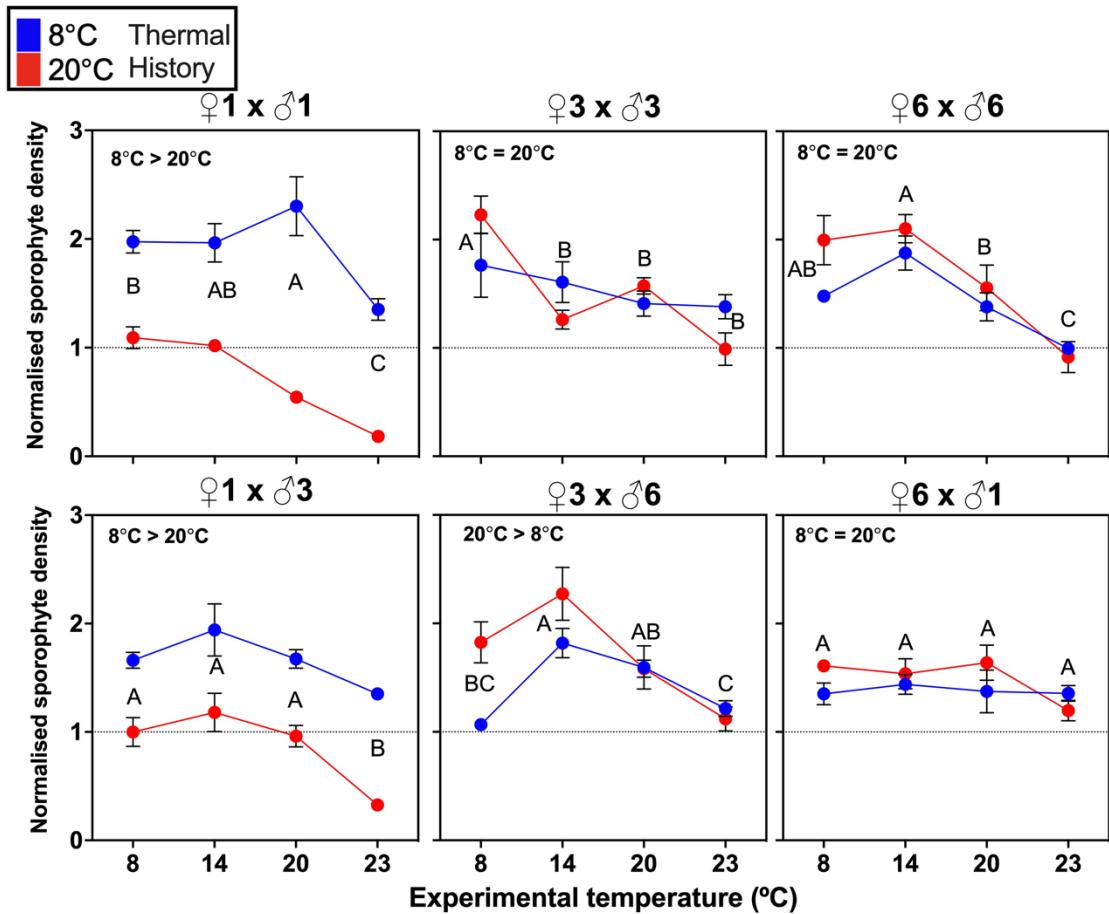
stable (normalised density  $\approx 1$ ) at the lowest temperatures (8 and 14°C), but decreased at higher temperatures, particularly at 23°C, indicating sporophyte mortality.

The normalised sporophyte density showed no significant cross x temperature x thermal history interactions, but all three two-factor interactions were significant (Table 7). Parental thermal history had no influence on the sporophyte density of both crosses with ♀6 and in the inbred cross ♀3 × ♂3, while higher sporophyte densities (2.3-fold) were observed in the two crosses with ♀1 with a LTH (Fig 8). In contrast, a HTH enhanced sporophyte density (1.2-fold) in the outcrossed ♀3 × ♂6 compared to a low parental thermal history. Overall, lower sporophyte densities (1.6-fold) were observed at 23°C irrespective of the thermal history than at all the other temperatures (except in the cross ♀6 × ♂1 where the sporophyte density did not significantly vary between experimental temperatures). On the other hand, the highest densities in general were found at 14°C.

**Table 7.** ANOVA for the effects of cross, temperature and thermal history on the density of sporophytes of *Laminaria pallida* after 16 days in experimental conditions. The post-hoc results are presented in Fig. 8.

<b>Factor</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Cross	5	2.62	0.52	6.548	<b>&lt;0.001</b>
Temperature	3	11.57	3.86	48.20	<b>&lt;0.001</b>
Thermal history	1	2.63	2.63	32.90	<b>&lt;0.001</b>
Cross × Temperature	15	4.74	0.32	3.95	<b>&lt;0.001</b>
Cross × Thermal history	5	14.74	2.95	36.84	<b>&lt;0.001</b>
Temperature × Thermal history	3	1.99	0.67	1.42	<b>&lt;0.001</b>
Cross × Temperature × Thermal history	15	1.70	0.11		0.15
Residual	144	11.52	0.08		

Significant interactions or main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 8: Effects of parental thermal history and temperature on the sporophyte density of different crosses after 16 days in experimental conditions.** Connected mean plots with standard error of the mean ( $n = 4$ ). Each plot corresponds to a cross of parental gametophytes. For each cross, different letters indicate differences between experimental temperatures irrespective of thermal history. Differences between thermal histories irrespective of experimental temperatures are noted in the upper left corner of graphs ( $p < 0.05$ ). See Table 7 for statistics.

### Sporophyte photosynthetic ability

Significant cross  $\times$  temperature  $\times$  thermal history interactions were detected for both maximum photosynthetic yield ( $F_v/F_m$ ) and relative maximum electron transport rate (rETRmax, Table 8). Overall, normalised  $F_v/F_m$  and rETRmax values were significantly higher (1.15-fold and 1.7-fold, respectively) in HTH sporophytes. This was especially prevalent for the sporophytes exposed to the highest temperature of 23°C (Table 9, Figs. 9 and 10). Only in the inbred cross  $\text{♀} 6 \times \text{♂} 6$ , a LTH resulted in higher sporophyte  $F_v/F_m$  at 8°C compared to HTH (1.15-fold).

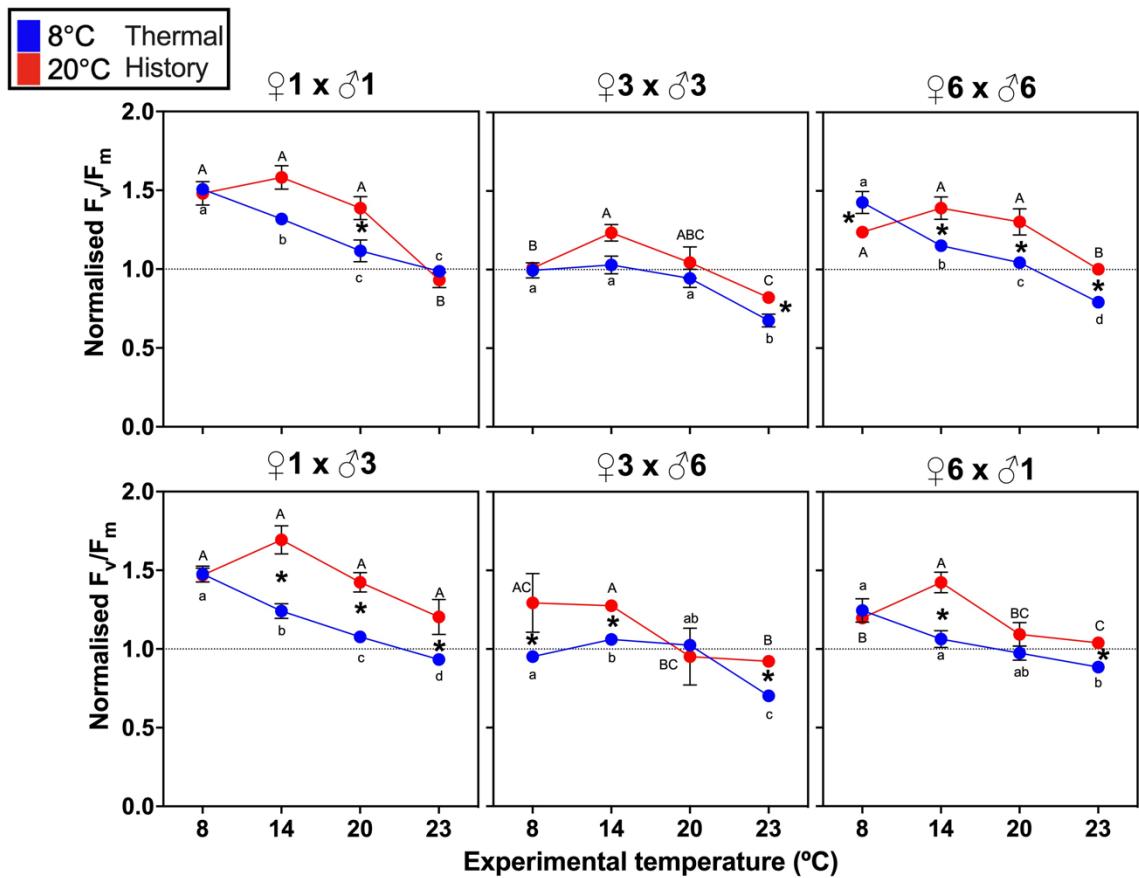
In general, the highest  $F_v/F_m$  values were observed at 8°C for the sporophytes with a LTH, while it was at 14°C for the HTH (Fig. 9).  $F_v/F_m$  was negatively affected by increasing experimental temperatures, being more evident in the sporophytes with a LTH. At the highest temperature of 23°C,  $F_v/F_m$  significantly decreased compared to all the other temperatures in the sporophytes from both thermal histories (1.4-fold).

Overall, the highest rETRmax values occurred at 8°C, regardless of the sporophyte parental thermal history (Fig. 10). For both thermal histories, rETRmax of sporophytes significantly declined at 23°C compared to all the other temperatures (1.7-fold), while for HTH sporophytes, this parameter was negatively affected already at 20°C compared with the lower temperatures (1.5-fold decrease).

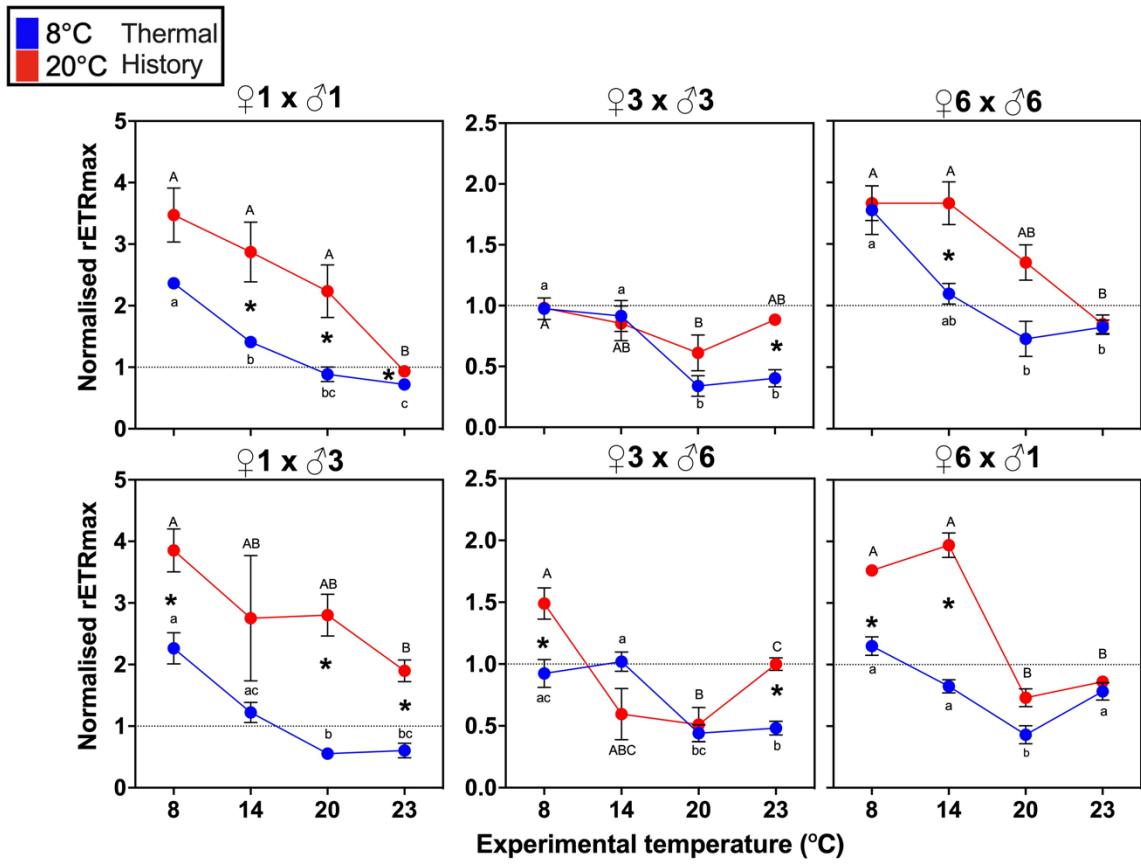
**Table 8.** PERMANOVA for the effects of cross, temperature and thermal history on the photosynthetic efficiency of *Laminaria pallida* sporophytes after 16 days. The post-hoc results are presented in Figs. 9 and 10 and Table 9.

Factor	df	SS	MS	Pseudo - F	P(perm)
Normalised $F_v/F_m$					
Cross	5	3.09	0.62	38.63	<b>0.0001</b>
Temperature	3	4.53	1.51	94.49	<b>0.0001</b>
Thermal history	1	1.20	1.20	74.90	<b>0.0001</b>
Cross × Temperature	15	0.38	0.03	1.57	0.0901
Cross × Thermal history	5	0.13	0.03	1.66	0.1453
Temperature × Thermal history	3	0.46	0.15	9.50	<b>0.0002</b>
Cross × Temperature × Thermal history	15	0.63	0.04	2.64	<b>0.0016</b>
Residual	144	2.30	0.02		
Normalised rETRmax					
Cross	5	44.89	8.98	44.45	<b>0.0001</b>
Temperature	3	33.35	11.12	55.04	<b>0.0001</b>
Thermal history	1	20.81	20.81	103.03	<b>0.0001</b>
Cross × Temperature	15	12.16	0.81	4.01	<b>0.0001</b>
Cross × Thermal history	5	13.77	2.75	13.64	<b>0.0001</b>
Temperature × Thermal history	3	0.93	0.31	1.55	0.2049
Cross × Temperature × Thermal history	15	5.76	0.38	1.90	<b>0.0264</b>
Residual	144	29.08	0.20		

Significant interactions or main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 9: Effects on parental thermal history and temperature on the sporophyte maximum photosynthetic yield ( $F_v/F_m$ ) of different crosses after 16 days in experimental conditions.** Connected mean plots with standard error of the mean ( $n = 4$ ). Each plot corresponds to a cross of parental gametophytes. \* indicates a significant difference between thermal histories per cross and experimental temperature. For each cross and each thermal history, different letters indicate differences between experimental temperatures (uppercase letters for 20°C thermal history and lowercase for 8°C thermal history,  $p < 0.05$ ). See Table 8 for statistics.



**Figure 10:** Effects of parental thermal history and temperature on the sporophyte relative maximum electron transport rate (rETRmax) of different crosses after 16 days in experimental conditions. Connected mean plots with standard error of the mean ( $n = 4$ ). Each plot corresponds to a cross of parental gametophytes. \* indicates a significant difference between thermal histories per cross and experimental temperature. For each cross and each thermal history, different letters indicate differences between experimental temperatures (uppercase letters for 20°C thermal history and lowercase for 8°C thermal history,  $p < 0.05$ ). See Table 8 for statistics.

**Table 9.** Summary of the significant differences between thermal histories (TH) per experimental temperature in the photosynthetic parameters of *Laminaria pallida* sporophytes.

Photosynthetic parameter	$F_v/F_m$		rETRmax	
	8°C TH > 20°C TH	20°C TH > 8°C TH	8°C TH > 20°C TH	20°C TH > 8°C TH
Significant differences				
8°C	1	1	0	3
14°C	0	4	0	3
20°C	0	3	0	2
23°C	0	5	0	4

## Sporophyte growth

Over the first 8 days in experimental conditions, the absolute growth rate (AGR) of sporophytes showed a significant interaction between all factors (cross x temperature x thermal history; Table 10). In general, sporophytes grew significantly faster (1.6-fold) when

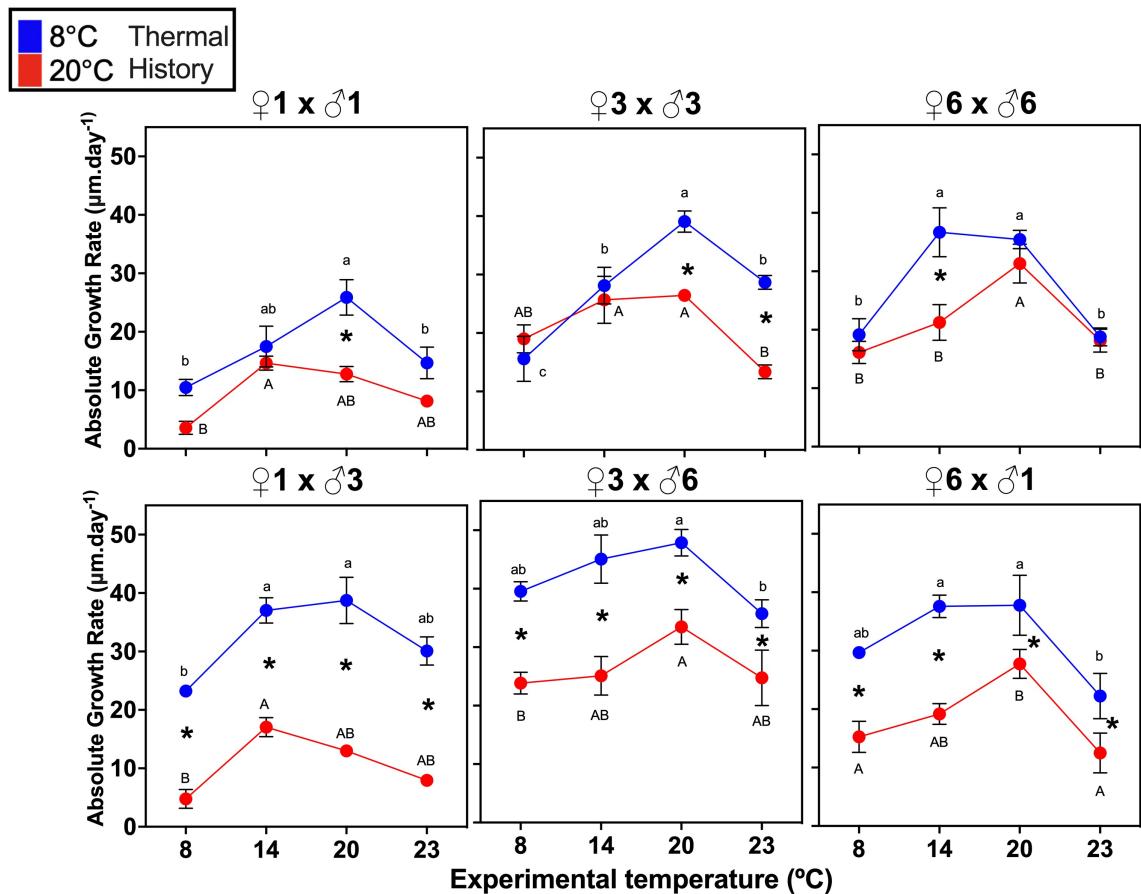
parental gametophytes were exposed to low temperatures compared to high temperatures exposure (Fig. 11, Table 11), except for the outcrosses  $\text{♀} 1 \times \text{♂} 3$  and  $\text{♀} 3 \times \text{♂} 6$  at 8°C and 14°C and for the  $\text{♀} 6 \times \text{♂} 1$  at 20°C and 23°C, where the parental thermal history had no influence in the sporophyte growth rate. In the sporophytes from both parental thermal histories, growth rates were generally highest at 20°C, except for  $\text{♀} 1$  crosses that were highest at 14°C. Overall, the AGRs of high and low parental thermal history were significantly lower at lowest experimental temperature (8°C, 1.6-fold) and at the highest temperature (23°C, 1.5-fold) than at 14°C and 20°C. Within crosses and thermal histories, the patterns were similar in crosses sharing a female gametophyte parent.

Over the next 8 days, the absolute growth rate differed due to cross  $\times$  temperature and cross  $\times$  thermal history interactions (Table 10). Thermal history effects were linked to maternal gametophytes: in the  $\text{♀} 1$  crosses, higher growth rates (1.3-fold) were observed in the LTH sporophytes, whereas in the  $\text{♀} 3$  crosses the growth was enhanced (1.2-fold) in the sporophytes with high thermal history compared with LTH, and finally the sporophytes from  $\text{♀} 6$  crosses showed no significant differences on the growth rate between parental thermal histories (Fig. 12, Table 11). Overall, sporophyte growth rates were highest at 14°C, significantly decreasing at the lowest temperature tested of 8°C (1.3-fold; in some crosses also at 20°C) and even more at the highest temperature (23°C, 2.9-fold) from day 8 to day 16. Overall, AGRs during the first 8 days were lower than from day 8 to day 16 (*c.f.* Figs. 11 and 12). The first time period showed an average growth rate of  $23.95 \mu\text{m day}^{-1}$ , with a maximum of  $53.06 \mu\text{m day}^{-1}$ , whereas the second time period averaged  $83.59 \mu\text{m day}^{-1}$  (3.5-fold increase) with a maximum of  $180.65 \mu\text{m day}^{-1}$  (3.4-fold increase). Growth rates increased more in sporophytes with a high (4.5-fold increase) compared with low (2.9-fold increase) thermal history.

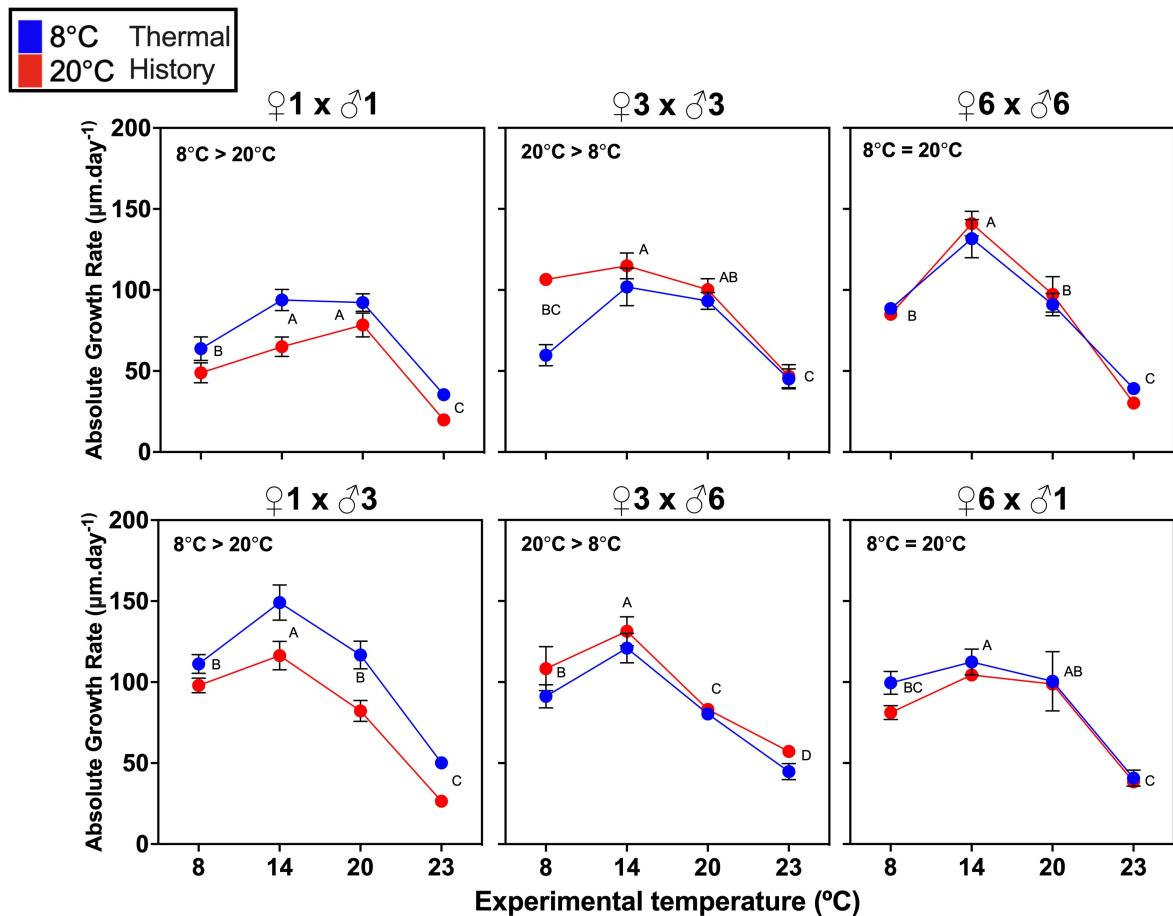
**Table 10.** ANOVA for the effects of cross, temperature and thermal history on the absolute growth rate based on length of sporophytes of *Laminaria pallida* after 8 and 16 days. The post-hoc results are presented in Figs. 11 and 12 and Table 11.

Factor	df	SS	MS	F	P
Absolute growth rate, d0-d8					
Cross	5	7306.75	1461.35	59.26	<0.001
Temperature	3	5131.29	1710.43	69.36	<0.001
Thermal history	1	6509.18	6509.18	263.96	<0.001
Cross × Temperature	15	624.48	41.63	1.69	0.059
Cross × Thermal history	5	1525.25	305.05	12.37	<0.001
Temperature × Thermal history	3	142.41	47.47	1.93	0.128
Cross x Temperature × Thermal history	15	930.61	62.04	2.52	0.002
Residual	144	3550.99	24.66		
Absolute growth rate, d8-d16					
Cross	5	19822.20	3964.44	23.00	<0.001
Temperature	3	145758.89	48586.30	281.90	<0.001
Thermal history	1	730.78	730.78	4.24	0.041
Cross × Temperature	15	14662.34	977.49	5.67	<0.001
Cross × Thermal history	5	11092.36	2218.47	12.87	<0.001
Temperature × Thermal history	3	613.22	204.40	1.19	0.317
Cross x Temperature × Thermal history	15	3725.04	248.34	1.44	0.136
Residual	144	24818.65	172.35		

Significant interactions or main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares.



**Figure 11: Effects of parental thermal history and temperature on the sporophyte absolute growth rate based on length of different crosses after 8 days in experimental conditions.** Connected mean plots with standard error of the mean ( $n = 4$ ). Each plot corresponds to a cross of parental gametophytes. \* indicates a significant difference between thermal histories per cross and experimental temperature. For each cross and each thermal history, different letters indicate differences between experimental temperatures (uppercase letters for 20°C thermal history and lowercase for 8°C thermal history,  $p < 0.05$ ). See Table 10 for statistics.



**Figure 12:** Effects of parental thermal history and temperature on the sporophyte absolute growth rate based on length of different crosses between 8-16 days in experimental conditions. Connected mean plots with standard error of the mean ( $n = 4$ ). Each plot corresponds to a cross of parental gametophytes. For each cross, different letters indicate differences between experimental temperatures irrespective of thermal history. Differences between thermal histories irrespective of experimental temperatures are noted in the upper left corner of graphs ( $p < 0.05$ ). See Table 10 for statistics.

**Table 11.** Summary of the significant differences between thermal histories (TH) per experimental temperature in the absolute growth rate of *Laminaria pallida* sporophytes.

Time period	0-8 days		8-16 days	
	8°C TH > 20°C TH	20°C TH > 8°C TH	8°C TH > 20°C TH	20°C TH > 8°C TH
8°C	3	0	2	2
14°C	4	0	2	2
20°C	5	0	2	2
23°C	4	0	2	2

## **Discussion**

This study showed intra- and transgenerational plasticity in *Laminaria pallida*. Gametophytes under prolonged exposure to 20°C (high thermal history, HTH) showed decreased physiological health compared to gametophytes exposed to 8°C (low thermal history, LTH). However, HTH gametophytes showed faster and more successful reproduction. In F1 sporophytes, HTH promoted increased photosynthetic efficiency at moderate to high temperatures (14-23°C), suggesting higher resilience and recovery ability. During the first 8 days in experimental conditions, LTH sporophytes were healthier and grew faster. During the second 8-days time period, effects of transgenerational plasticity on growth were linked to maternal genetic lines, suggesting HTH sporophytes had recovered from stress and were equally healthy as LTH sporophytes. Effects of thermal stress on growth and density were associated to maternal genetic line-specific transgenerational effects.

### **Decreased gametophyte health at high temperatures**

Gametophytes were less healthy when exposed to high temperatures (20°C) compared to 8°C. Although the sporophytes of *Laminaria pallida* are reproductive year-round, the peak is mainly at the end of the austral summer (March, Dieckmann, 1980), when surface temperatures are still warm (17-21°C in Swakopmund, (*Namibia Sea Temperatures*)). Gametophytes will settle in the rocky substrate under the parental canopy and most probably remain vegetative for several months until average temperatures allow gametogenesis ( $\leq$  17°C - May). Vegetative gametophytes in nature are therefore exposed to similar temperatures as the high temperature treatment (20°C) used in this study. In *Laminaria digitata*, Martins et al. (2020) observed decreases in gametophyte growth when exposed to temperatures near or at the upper survival limit. Additionally, Delebecq et al. (2016) reported decreases in photosynthetic efficiency of *L. digitata* at high temperatures, albeit during a

short experiment. Although 20°C is within the thermal range of *L. pallida*, tom Dieck (1993) observed that the upper survival temperature of gametophytes decreases with prolonged exposure, from 25-26°C to 23°C after 8 weeks. Our experimental conditions of 20°C for 3.5 months are therefore near the upper boundary. Gametophytes exposed to such temperatures *in situ* are therefore in sublethal conditions for extended periods of time, leading to decreased physiological health. The shapes of gametophyte clusters reinforce this conclusion. Indeed, while gametophytes at low temperatures formed lattices of filaments to maximise light and nutrient exposure, high temperatures lead to dense, coiled clusters to reduce exposure to experimental conditions. Outward, exposed filaments systematically presented numerous transparent, dead cells. *In situ* water motion conditions can prevent gametophytes from forming clusters protecting most cells and could lead to more significant degradation than in our study. The response of gametophytes to prolonged exposure to high temperatures indicates potential challenges linked to climate change. Increases in water temperatures and vegetative duration mean that gametophytes will be subject to sublethal conditions for longer durations. Northern populations of *L. pallida* are at risk of becoming extinct as gametophytes are extremely important for the persistence of kelp species.

In rarer cases, when sporophyte reproduction happens during colder months, the developed gametophytes can become reproductive (at low temperatures of 8°C, Martins et al. 2019) or may settle below the canopy under dark conditions remaining in a vegetative state for extended periods of time before irradiance is sufficient for them to become reproductive. In this case, the gametophytes remained healthy during prolonged exposure to cold temperatures. This suggests a higher acclimation to cold conditions than warm in gametophytes of *L. pallida*.

Strain specific responses were observed, with four strains showing lower  $F_v/F_m$  values than strains ♂1 and ♀6. These differences can be related with lack of nutrients, space and light

exposure due to higher gametophyte growth and cluster formation over in the initial weeks in the strains presenting lower photosynthetic efficiency.

#### *Thermal history has no influence on gametophyte growth*

Gametophyte growth at 14°C was not influenced by the thermal histories (8°C and 20°C). While we hypothesised that a high thermal history (HTH) would lead to increases in gametophyte growth, some factors can explain the results obtained. The transfer to 14°C was accompanied by exposure to white light, both of which are triggers of gametogenesis (Pearson et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993). As gametogenesis and growth are mutually exclusive processes in kelps, it is possible that potential resources linked to thermal history were assigned to the formation of reproductive structures (antheridia and oogonia) rather than growth.

#### **High thermal history promotes gametogenesis and recruitment**

Overall, we observed intragenerational carry-over effects of thermal history on gametophyte reproductive performance. A high thermal history promotes the speed of gametogenesis, the female gametophyte reproductive success and sporophyte recruitment in *L. pallida*. Although matching our hypothesis, these results contrast with previous experiments conducted in kelps. Lüning (1980) and Martins et al. (2020) observed that high thermal histories lead to decreases in the fertility of gametophytes of several cold-adapted kelp species, while Gauci (2020) reported increases in reproduction of *L. digitata* after exposure to cold temperatures. In the fish *Oryzias latipes*, high parental thermal history led to faster reproduction at lower temperatures (Loisel et al., 2019). However, no studies have been conducted on *L. pallida* or other warm-temperate kelp species. The high thermal history promotion of gametogenesis and recruitment agrees with its seasonal reproductive pattern in the native environment, as

spores are mainly released in the end of the summer leading to long-term exposure of the developed gametophytes to high temperatures during the summer/autumn months. Our hypothesis supposed that gametophytes experiencing such warm temperatures would become more reproductive and faster when exposed to favourable gametogenic conditions, which was confirmed by our results.

Gametogenesis in algae is known to be associated with specific environmental cues such as seawater temperature or irradiance (Martins et al., 2017; tom Dieck (Bartsch) & de Oliveira, 1993). Proximate stressors, such as water motion, can also act as key signals in gamete release. In fucoid algae, gamete release was observed only in still conditions to avoid excessive dilution of gametes and increase fertilisation success (Pearson et al., 1998; Pearson & Serrão, 2006; Serrao et al., 1996). In our case, the high thermal history stressor may also be an environmental cue, preconditioning gametophytes and leading to improved reproduction when cooler conditions followed. Pearson et al. (2019) observed specific gene activation when switching gametophytes of the kelp *Saccharina latissima* from red to white light, triggering gametogenesis. It is possible that the thermal history of gametophytes affects the rate or extent to which such genes are activated, impacting gametogenesis and reproductive success.

Additionally, some plants are reported to optimise their reproduction when faced with environmental stressors. Kazan & Lyons (2016) reported that plants of the genus *Arabidopsis* flower faster when exposed to a number of stressors, including heat. While studies are limited in algae, Suda & Mikami (2020) reported that heat stress led to the production of reproductive cells in mature *Pyropia yezoensis*. It is possible that similar effects of thermal stress further promoted subsequent reproduction during our experiment.

Results show that as climate change progresses, conditions may favour crosses with increased sporophyte output after exposure to high thermal history, potentially selecting more resilient genetic lines while decreasing diversity.

Although, LTH gametophytes showed lower reproductive ability during the gametogenesis experiment compared to HTH, drastic increases in sporophyte density in all LTH crosses were observed during the transgenerational experiment, with sporophytes from crosses with ♀ 1 almost doubling in density. These results suggest strong gametophyte reproduction during this subsequent period under different experimental temperatures. Gametophyte density is known to influence reproduction, with high densities impeding gametogenesis (Ebbing et al., 2020; Reed, 1990; Reed et al., 1991). For the transgenerational experiment, the gametophytes and the microscopic sporophytes developed in each replicate were split between four larger ones. The subsequent increase in space, light and nutrient availability may have facilitated gametogenesis in the less reproductive LTH gametophytes. When sporophyte reproduction happens during colder months, vegetative gametophytes are likely to be exposed to cold temperatures for extended periods of time. Our results suggest that such low thermal history leads to slower reproduction, potentially requiring increased light and nutrient availability than high thermal history.

Interestingly, a systematic increase in sporophyte densities was observed at all temperatures except at the highest temperature of 23°C. Even at 23°C, five out of six crosses with LTH showed slight increases in density. This contrasts with published results (tom Dieck (Bartsch) & de Oliveira, 1993) where gametophytes of *L. pallida* did not become reproductive at temperatures higher than 17°C. We hypothesise that after reproduction is triggered by favourable/permissive conditions, this pathway or developmental decision is not reversed, even by the return of gametophytes to unfavourable temperatures.

## **Transgenerational effects on juvenile sporophytes**

*L. pallida* microscopic sporophytes displayed transgenerational plasticity, at moderate to high temperatures. HTH increases the sporophytes photosynthetic efficiency at moderate to very high temperatures (14-23°C) compared to LTH. Additionally, thermal history shifts the temperature at which the sporophytes show the highest  $Fv/Fm$  values, from 8°C in LTH sporophytes to 14°C in HTH, with both thermal histories showing slight decreases at higher temperatures. These results differ with the literature which suggests photosynthetic yields generally increase with increasing temperatures up to sublethal limits where they tend to collapse rapidly (Burdett et al., 2019; Roleda, 2009, Delebecq et al., 2016). However, they do suggest that a high parental thermal history improves the health of juvenile sporophytes, especially at the temperatures experienced *in situ* (12-22°C in Swakopmund, *Namibia Sea Temperatures*).

In the crosses with ♀ 1, HTH sporophytes showed extreme increases in rETRmax when exposed to 8°C. Such results suggest a drastic increase in sporophyte health over the 16 days of the experiment, most likely due to poor physiological condition at the beginning, which improved when sporophytes were transferred to conditions with increased nutrient access and space. Additionally, the sporophytes were visually very pale to transparent at the beginning of the experiment, indicating poor health. This hypothesis is corroborated by density results, where these crosses were the only ones exhibiting significant mortality at the stressful temperature of 23°C.

Sporophyte growth showed different patterns during the two time periods. Over the first 8 days, LTH sporophytes grew significantly more than HTH ones at mild to warm temperatures (14-20°C). However, thermal plasticity during the second time period (from day 8 to day 16) was dependent on maternal genetic line only, while absolute growth rates increased drastically. These results suggest that the sporophytes were adapting to the new experimental

conditions during the first time period, before reaching normal growth rates. The initially higher growth rates in LTH sporophytes suggest that all HTH sporophytes may have initially been in poorer health condition, with different degrees of vulnerability and recovery potential highlighted by density results. Such initial fragility of sporophytes may be linked to the increased reproduction observed for HTH gametophytes, where suboptimal resources may be allocated to each offspring.

Our results therefore suggest that HTH leads to fragile sporophytes, which are more likely to be impacted by high temperature stressors. In an ecological context, this fragility may not be very significant. Indeed, HTH arises when gametophytes are released at the end of the Austral summer and are vegetative during the autumn. In turn, sporophyte recruitment happens early during winter, and juvenile sporophytes are exposed to mild temperatures during the first months of growth (12-16°C, Dieckmann, 1978). In our experiment, HTH sporophytes exposed to 14°C showed high photosynthetic efficiency and fast recovery (8 days), after which growth rates were similar or higher to LTH sporophytes.

In dinoflagellate coral symbionts, pre-exposure to high temperatures led to an improved efficiency of photosynthetic apparatus during and after heat waves (Middlebrook et al. 2008). As a result, survival and recovery of the corals and their symbionts were drastically improved. We observed that HTH led to higher photosynthetic efficiency than LTH in sporophytes of *L. pallida*, potentially helping the fragilised HTH sporophytes recover. If such transgenerational effects are maintained over the first few months of sporophytes growth, they could increase resilience and recovery of sporophytes to heat stress experienced during summer months.

On the other hand, when gametophyte release happens during colder months, leading to low thermal histories, juvenile sporophytes are likely to face warmer temperatures in early life stages. Our results suggest that such gametophytes are originally healthier and show good

survival even at very high temperatures. The decreases in photosynthetic efficiency, however, suggest a relatively low physiological resilience to heat stress.

### **Juvenile sporophytes thermal acclimation**

Sporophyte growth at very high temperatures suggests that the negative effects of heat stress increase over time. During the first time period, growth rates were already significantly lower (1.5-fold) at 23°C compared to mild-warm temperatures (14-20°C), showing effects of stress. However, the difference almost doubled during the second time period (2.9-fold). This rapid increase suggests either deteriorating health of sporophytes or increased allocation of resources to survival rather than growth. Photosynthetic and density results also pointed to increased stress at 23°C. Prolonged exposure to heat stress therefore reduces acclimation and resilience to such stressors. While short heat waves may not significantly affect the growth, it appears that longer exposure has negative effects, in concordance with other studies in *L. pallida* (Martins et al., 2019; tom Dieck (Bartsch) & de Oliveira, 1993; tom Dieck, 1993). Additionally, if sporophytes survive such long-term heat stress, the resulting stunted growth might decrease their competitiveness and recovery ability in natural conditions.

### **Effects of genetic lines on plasticity**

Evident female effects were detected in gametophyte reproduction. Patterns of gametogenesis were very similar for crosses with the same female strain and thermal history over time. During reproduction, carry-over effects of thermal history were also affected by the female genetics. Indeed, females 1 and 3 showed almost ubiquitous intragenerational plasticity while ♀ 6 showed little to none. This variation both in reproduction and ability to maintain plasticity within a generation may be linked to the aptitude to form and retain epigenetic markers such as DNA methylation. Similar genetic variation in plasticity has been observed

in plants (Sultan et al., 2009; Vu et al., 2015). Similarly, transgenerational plasticity of juvenile sporophytes was affected by maternal genetics. Particularly, sporophytes growth rates showed different transgenerational effects for each maternal line, suggesting a strong impact of genetic material on the aptitude for transgenerational plasticity. Liesner et al. (2020) also reported in *Laminaria digitata* different results of thermal transgenerational plasticity for each genetic line studied. These results suggest that DNA methylation is retained more during maternal egg formation than paternal gametogenesis. Such maternal effects on transgenerational plasticity have been observed in a variety of taxa (e.g., Galloway & Etterson, 2007; Marshall, 2008; Shama et al., 2014) and are considered the norm, as females have more ways to impact offspring development (e.g., egg provisioning, resource sharing when the offspring is attached). No significant paternal effects were observed during our study.

No noticeable differences between inbred and outcrossed crosses were detected in gametophyte reproduction and juvenile sporophyte rearing. While self-fertilisation is generally possible in kelps, it has been shown to cause large decreases in fitness and plasticity in offspring of the giant kelp *Macrocystis pyrifera* (Raimondi et al., 2004). However, in smaller, isolated populations of *Postelsia palmaeformis*, inbreeding did not impact the thermal tolerance of the offspring (Barner et al., 2011). The studied population, located in Northern Namibia, is also relatively isolated. Recent research by Assis et al. (in prep.) suggests that this population has very high inbreeding rates. It is therefore likely that all crosses, whether inbred or outcrossed, shared significant genetic material, thus reducing our capacity to observe inbreeding effects against a potentially inbred background. The low genetic diversity may decrease the potential for genetic adaptations to arise when facing new stressors.

The genetic variation of transgenerational plasticity is double-edged. The genetic component of plasticity offers a target for selection, potentially increasing the resilience and fitness of entire populations (Chevin et al., 2010; Munday et al., 2017, 2019). On the other hand, such selection is likely to lead to a loss of genetic variation. In remote, sparse populations such as the one studied, this might lead to problematic inbreeding rates. However, further studies would be necessary to evaluate potential negative effects of inbreeding as our results here are inconclusive.

## Conclusion

*L. pallida* gametophytes showed lower photosynthetic efficiency after ~3.5 months at 20°C than at 8°C, suggesting lower tolerance to long-term heat stress. However, a high thermal history led to faster, more successful gametophyte reproduction, which may be a product of the natural seasonality of reproduction and/or of stress-induced gametogenesis. Juvenile sporophytes were also affected by the thermal history of parental gametophytes. A high thermal history led to improved photosynthetic efficiency, especially at high temperatures. On the other hand, juvenile sporophytes from a high thermal history showed decreased growth and adaptability when placed in new conditions (thermal gradient), suggesting they were less physiologically robust than those with a low thermal history. However, after adapting to new conditions, the effects of thermal history on the growth of juvenile sporophytes were linked exclusively to maternal genetic lines. Such effects of genetic material on intra- and transgenerational plasticity were detected in several crosses during both gametophyte reproduction and juvenile sporophyte growth, suggesting a strong genetic variation of intra- and transgenerational plasticity. Irrespective of thermal history or genetic lineage, F1 sporophytes showed optimal photosynthetic ability and growth at medium to

warm temperatures (14-20°C), with a sharp decline in fitness at the upper survival temperature of 23°C.

Overall, *Laminaria pallida* vegetative gametophytes showed acclimation to high growth/maintenance temperatures, with positive subsequent intragenerational effects on reproductive output. Fitness-related traits in F1 sporophytes indicated that transgenerational changes in plasticity occurred, with high thermal histories leading to increased photosynthetic efficiency at high temperatures and increased growth in some genetic lines. Further investigation is needed to understand the potential benefits for natural populations and interactions with climate change-driven stressors.

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