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THE ROLE OF SEASONALITY IN REPRODUCTION OF MULTIANNUAL DELAYED GAMETOPHYTES OF *SACCHARINA LATISSIMA*[1]

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Delayed gametophytes are able to grow vegetatively for prolonged periods of time. As such, they are potentially very valuable for kelp aquaculture given their great promise in opening up novel opportunities for kelp breeding and farming. However, large-scale application would require more in-depth understanding of how to control reproduction in delayed gametophytes. For newly formed gametophytes, many environmental factors for reproduction have been identified, with key drivers being light intensity, temperature, and the initial gametophyte density. However, the question of whether delayed gametophytes react similarly to these life cycle controls remains open for exploration. In this study, we performed a full factorial experiment on the influences of light intensity, temperature, and density on the reproduction of multiannual delayed gametophytes of *Saccharina latissima*, during which the number of sporophytes formed was counted. We demonstrate that delayed gametophytes of *S. latissima* can reliably reproduce sexually after more than a year of vegetative growth, depending on the effects between light intensity and temperature. Under higher light intensities (≥29 µmol photons · m^{-2} · s^{-1}), optimal reproduction was observed at lower temperatures (10.2°C), while at lower light intensities (≤15 µmol photons · m^{-2} · s^{-1}), optimal reproduction was observed at higher temperatures (≥12.6°C). Given the seasonal lag between solar radiation and sea surface temperature in natural systems, these conditions resemble those found during spring (i.e., increasing light intensity with low temperatures) and autumn (i.e., decreasing light intensity with higher temperatures). Seasonality can be used as an aquaculture tool to better control the reproduction of delayed gametophytes.

Key index words: **initial gametophyte density; kelp; life cycle control; light intensity; seasonal lag; temperature; vegetative growth**

***Abbreviations*: CRM, controlled reproductive method; URM, uncontrolled reproductive method**

Crop domestication requires humans to understand and exert control over the life cycles of target organisms (Zeder 2015) and involves building multigenerational relationships (Valero et al. 2017). Vodouhè and Dansi (2012) describe seven cultivation phases in the domestication process of crops (Table S1 in the Supporting Information). Kelp aquaculture currently hovers somewhere between phase 4 and phase 6 of these cultivation phases. Phase 4 is the phase in which the reproductive biology of the species is known but mass production still needs to be mastered. Phase 5 is when the crop is cultivated and harvested using traditional practices. Phase 6 is the phase where farmers are able to adopt specific criteria to select plants that better satisfy the needs of consumers, and even though some kelp farmers are already selecting plants that are able to better satisfy the needs of consumers (Valero et al. 2017, Zhang et al. 2017), many phase 4 aspects of mass production are still researched today. These include identifying the most suitable date of planting (Peteiro and Freire 2009), substrate optimization (Kerrison et al. 2018), year-round zoospore production (Forbord et al. 2012), identifying optimal farming locations (Matsson et al. 2019), and

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development of disease management (Peng and Li 2013). For further progress in the domestication process of kelp, we need to increase our fundamental understanding of the life cycles of kelp and apply this knowledge in improving the cultivation methods.

To date, two different cultivation methods are practiced in kelp aquaculture (Valero et al. 2017, Forbord et al. 2018, Goecke et al. 2020): the uncontrolled reproductive method (URM) and the controlled reproductive method (CRM). In traditional URM (Shan et al. 2013), substantial numbers of zoospores are released in large tanks. These zoospores first attach on strings and develop into gametophytes, after which the newly formed gametophytes form gametes. The female gametophytes become fertilized, subsequently forming juvenile sporophytes, which then also attach to these strings (Su et al. 2017), all without direct intervention of farmers. In CRM, gametophyte reproduction is directly controlled under in vitro laboratory conditions (Ratcliff et al. 2017, Forbord et al. 2020). The induced gametophyte cultures, often already containing juvenile sporophytes, are then attached to seed strings using a method called paintbrush seeding (Redmond et al. 2014) or deployed immediately through direct seeding (Kerrison et al. 2018, Forbord et al. 2020). CRM is more labor-intensive than URM and results in lower attachment to seed strings (Xu et al. 2009). This probably explains why the URM is still commonly practiced in many parts of the world (Goecke et al. 2020).

In the future, using CRM might be more promising because (i) gametophytes can be kept in cultures for prolonged periods of time (Carney 2011, Barrento et al. 2016, Wade et al. 2020) and successfully cryopreserved (Visch et al. 2019), (ii) gametophyte reproduction still has room to be further optimized (Ratcliff et al. 2017), and (iii) this opens up new possibilities for breeding, by allowing for the crossing of distantly related unialgal male and female gametophyte clone cultures to increase intraspecific hybrid vigor in future F1 hybrid cultivars through heterosis (Shan et al. 2016, Zhao et al. 2016). It should be noted that the reliable use of gametophyte clones for large-scale aquaculture or breeding purposes can only be achieved by greater understanding of reproduction in larger volumes of delayed gametophyte cultures (Zhang et al. 2008). Presently, the great majority of gametophyte studies have been carried out using newly formed gametophytes (Bartsch et al. 2008). Large-scale application of CRM necessitates working with gametophytes that have delayed their sexual reproduction by growing vegetatively through mitosis as undifferentiated filamentous cells for prolonged periods of time (Pang and Wu 1996, Carney 2011, Zhang et al. 2019). Gametophytes can even delay reproduction for multiple years (Barrento et al. 2016), transgressing seasonally induced reproduction; these are hereafter referred to as multiannual delayed gametophytes. Hence, there is a need for a more in-depth understanding of how abiotic and biotic factors can trigger life cycle transitions and reproductive success in delayed gametophytes (Edwards 2000), particularly in multiannual delayed gametophytes, which is the focus of this study.

Members of the kelp order Laminariales have heteromorphic life cycles that transition between microscopic, haploid gametophytes and macroscopic, diploid sporophytes. This results in the spatiotemporal separation of meiosis and fertilization, with somatic development occurring at both the haploid and diploid stages (Bell 1997). The transitions between these life phases are controlled by abiotic and biotic environmental factors (Lüning and Dieck 1989), hereafter described as life cycle controls (Ebbing et al. 2020). Extensive studies on life cycle controls for newly formed *Saccharina latissima* gametophytes have identified the following as the main drivers: light intensity (Hsiao and Druehl 1971, Bolton and Levitt 1985), temperature (Lüning and Neushul 1978, Morita et al. 2003), nutrients (Harries 1932, Martins et al. 2017), daylength (Choi et al. 2005, Martins et al. 2017), and initial gametophyte density (Choi et al. 2005, Carney and Edwards 2010, Ebbing et al. 2020).

Given the importance of light and temperature in reproductive timing and success, growth and reproduction in most Laminariaceae are strongly seasonal, since they are primarily found in subtidal areas at higher latitudes, where light and temperature vary strongly over the year (Krumhansl et al. 2016). Gametophyte reproduction in natural populations has been observed throughout the year, making it likely that there are multiple times during the year when conditions are suitable for gametophyte reproduction (Parke 1948, Druehl 1965, Tatsumi 2018). The influence of seasonality in the reproduction of newly formed gametophytes has been thoroughly studied and indicates that interactions between light and temperature greatly influences the growth and reproduction of newly formed *Saccharina latissima* gametophytes (Lüning 1980, Lee and Brinkhuis 1986, 1988, Park et al. 2017). This dynamic between light and temperature can be seen all around the world, with the yearly cycle of sea surface temperature always lagging behind the yearly cycle of daily radiation in the northern hemisphere resulting in increasing light intensities with low sea surface temperatures during spring and decreasing light intensities with high sea surface temperatures during autumn (Donohoe 2020). This consistent lag between these two abiotic factors might potentially function together as an important life cycle control mechanism within the life cycles of kelp. We hypothesize that multiannual delayed *S. latissima* gametophytes use this lag between the seasonal cycles of solar irradiation and sea surface temperature as a strong life cycle control in order to align

gametogenesis with sorus formation, thus aligning gametogenesis to the dispersal of large quantities of zoospores. In addition, initial gametophyte density is included in this experiment, as it has been shown to be a strong biotic life cycle control mechanism in higher density gametophyte cultures (Reed 1990, Choi et al. 2005, Carney and Edwards 2010, Ebbing et al. 2020).

METHODS

Culture collection. Ripe *Saccharina latissima* individuals were collected along the coast of Flekkefjord, Norway, (58.294270° N, 6.656618° E) in December 2016. The ripe sori of ten pooled individuals were excised from the blades, and biofouling was thoroughly removed using absorbent paper. The collected sori were further cleaned by shortly (~1 min) submerging them in hypochlorite 0.15% (ClO^-), followed by rinsing three times with pasteurized seawater. The cleaned sori were then dried using absorbent paper, followed by overnight drying in an incubator (12°C). Zoospores were released the next day in Erlenmeyer flasks, by submerging the sori in seawater, and these cultures were hereafter incubated at 12°C under red light (30 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, 12:12 h light:-dark cycle) in f/2 medium (Guillard and Ryther 1962). These cultures were then incubated for 1.5 y prior to the start of the experiment. During this period, the medium was refreshed on a monthly basis and cultures were monitored for general health, infections, preliminary reproduction, and growth.

Gametophyte culture measurements. During the experiment, we used a dilution gradient of a stock solution to reach specific gametophyte biomass (0.01, 0.02, 0.04, 0.08 mg $\cdot$ mL^{-1}). In order to estimate initial gametophyte densities, we measured chlorophyll-a concentrations (Chl-*a*) with fluorometry (FastOCEAN/Act2 FRRF, Chelsea Technologies Group Ltd) and calibrated these against gametophyte biomass (Fig. S1 in the Supporting Information; $R^2 = 0.975$), similar to what has previously been done for phytoplankton biomass (Huot et al. 2007). The small volumes of the wells used in this experiment made it necessary to use biomass extrapolations of larger volumes of stock gametophyte culture (60 mL) to calculate relationships between fluorometry measurements and freeze-dried gametophyte dry weights (DW) that were weighted using a precision scale.

Modeled daily radiation and sea surface temperature. The seasonal cycles of daily radiation and sea surface temperature were collected close to the original environment of the parental sporophytes from publicly available datasets. These measurements were used to demonstrate the seasonal lag between light intensity and sea surface temperature, which characterizes the seasonal shift between spring and autumn. Modeled solar radiation derived from a climatology model (ERA INTERIM) represents 20-year averages (1993–2013) of a grid cell close to the origin of the parental sporophytes (57° N, 5.25° E). Note that estimates of mean surface solar radiation (kJ $\cdot$ m^{-2} $\cdot$ h^{-1}) were not modeled using daily radiation but the 20-y average of peak solar radiation between 10:00 and 14:00 so that they could be directly translated into in vitro light intensities instead of acting as a proxy for day length. The sea surface temperature data consists of five-year averages of the North Sea and come from the weather station located at the Ecofisk oil field (56.5434° N, 3.2244° E).

Light and temperature conditions. The variations in light intensity were achieved through specific placement of the cultures with respect to light sources. The manual placement of the well plates resulted in slightly different light intensities between these incubators, which was taken into account in the analyses afterward. Light intensities were measured at three points in the middle of the plate, after which an average light intensity was calculated per plate. The spectral distribution of white light was measured using a modular multispectral radiometer (TriOS Ramses ARC, Germany; Heuermann, Reuter and Willkomm 1999; Fig. S2 in the Supporting Information). The temperature range in this experiment was achieved using six small-scale incubators (Polar CE202; Fig. S3 in the Supporting Information). All incubators were adjusted for a specific temperature, with 10.4°C being the lowest reliable temperature that could be achieved using this setup without strong temperature gradients forming inside the incubators. LED panels (LS LED, RGB +CCT 30 *30 cm 18W) were placed in front of the glass door at an angle of approximately 45°. Randomly filled well plates ($n = 24$) were placed in each incubator along a light intensity gradient (10, 30, 60, and 80 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}).

Gametophyte reproduction. Successful reproduction was determined on day 28, and quantified using a proxy for gametogenesis, namely the number of sporophytes (≥25 μm) per mL. To elaborate further, our focus is the gametophytic part of kelp reproduction, where kelp reproduction as a whole consists of two spatiotemporally separated reproductive events (sporophytic meiosis and gametophytic fertilization). The sporophytes developed on the bottom of the well plates, and were counted in triplicate for each unique treatment ($n = 96$). After 28 d, all fertilized oogonia had developed into small sporophytes, which were still small enough to accurately distinguish for counting single individuals.

Statistical analysis. All statistical analysis was done using the SPSS 20.0.0 statistical package (SPSS Inc., Chicago, IL, USA), R version 3.6.0 (R Core Team 2018), and SigmaPlot 13.0 (Systat software Inc., London, UK). A linear regression using a 2nd order polynomial (parabola) was fitted to quantify the effects of temperature on reproductive success under two contrasting light regimes using R version 3.6.0. The reproductive success was log-transformed before fitting these regressions so as to avoid passing through 0, as predictions of negative reproduction are meaningless. Reproductive success was log-transformed to achieve normal distributions and analyzed for homogeneity of variance using Levene's test. In case of unequal variances, a robust test of equality of means for unequal variances was applied (Welch *t*-test). Games–Howell nonparametric post hoc comparisons were subsequently applied to test for significant differences between the subgroups (light intensities, temperature, and initial gametophyte densities). All tests were run with a significance level of 0.05. Data on the reproductive success of gametophytes ($n = 288$) are presented as means ± SD. Contour plots were also used to visualize the interactive influence of light intensity and temperature on the reproduction of multiannual delayed gametophytes at an initial gametophyte density of 0.01 mg $\cdot$ mL^{-1} (Loess smoother, sampling portio $n = 0.8$, interval = 6).

RESULTS

The interactive effect of light intensity, temperature, and initial gametophyte density on gametophyte reproduction. Reproduction of multiannual delayed *Saccharina latissima* gametophytes was successfully induced under different light intensities (μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), temperatures (°C), and initial gametophyte densities (mg $\cdot$ mL^{-1}). In Figure 1A, we plotted the optima of the reproductive success (sporophytes$\cdot mL^{-1}$) found at varying temperatures,

along with the corresponding initial gametophyte densities. The highest reproduction success observed in this experiment was 469 sporophytes · mL^{-1}, which was achieved at 8.5 µmol photons · m^{-2} · s^{-1}, 12.6°C, and at an initial gametophyte density of 0.04 mg · mL^{-1}. The effect of light intensity on reproduction is shown in Figure 1B for an initial gametophyte density of 0.01 mg · mL^{-1}. We only visualized results for the lowest initial gametophyte density (0.01 mg · mL^{-1}), as the interactive effects of light and temperature on gametophyte reproduction followed similar trends for all initial gametophyte densities, but were difficult to visualize on the same scale. This was mainly because higher initial gametophyte densities had strong negative interactive effects on gametophyte reproduction, thereby visually masking the relative influence of light and temperature on gametophyte reproduction (Fig. 2). The contour plots in Figure 1B show that the gradient of gametophyte reproduction success varied with light intensity, depending on the temperature that was used. At 10.4°C, the reproductive optimum was found at 60 µmol photons · m^{-2} · s^{-1}, while at ≥12.6°C the reproductive optima were found at lower light intensities (9–15 µmol photons · m^{-2} · s^{-1}). At a temperature of 11.2°C, an intermediate reproductive gradient was observed. Although light intensity did generally influence gametophyte reproduction (Welch ANOVA, $F_{3,4.07} = 7.483$, $P < 0.05$), no significant differences in reproductive success were found between light intensities (12.5, 29.0, and 58.0 µmol photons · m^{-2} · s^{-1}; Games–Howell, $P > 0.05$; Table S2 in the Supporting Information). Cultures with no gametophyte reproduction were also observed in this experiment (dark-blue color) and usually occurred at temperatures ≥14.0°C or at initial gametophyte densities >0.04 mg · mL^{-1} (Fig. 2).

The effect of initial gametophyte density on gametophyte reproduction. The relative reproductive success (sporophytes · mg^{-1}) was calculated by dividing the sporophytes that had formed (sporophytes · mL^{-1}) with the initial gametophyte density that had been used (mg · mL^{-1}). Increasing initial gametophyte density resulted in low relative reproduction success (Fig. 2). Optimal relative reproductive success was found at the lowest initial gametophyte density used in this experiment of 0.01 mg · mL^{-1}, with three optima of similar reproductive success (18.247 sporophytes · mg^{-1} ± 604) at three different temperatures (10.4, 11.2, and 12.6°C), depending on the light intensity used. This inverse correlation between initial gametophyte density and relative reproductive success was especially pronounced at higher light intensities (≥29 µmol photons · m^{-2} · s^{-1}), with relative sporophyte production at an initial gametophyte density of 0.08 mg · mL^{-1} mostly present at low light intensities (≤15 µmol photons · m^{-2} · s^{-1}).

Seasonality in multiannual delayed gametophyte reproduction. The relation between reproductive success and temperature was fitted with a polynomial linear regression for low (≤15 µmol photons · m^{-2} · s^{-1}) and high (≥29 µmol photons · m^{-2} · s^{-1}) light intensities (Fig. 3). Distinct optimal temperatures were found for reproduction under high and low light intensity regimes (ANCOVA, $F_{5,49} = 49$, $R^2 = 0.82$, $P \leq 0.001$; Table S3 in the Supporting Information). A similar disparity between light and temperature was also measured in a natural system, reflecting the seasonal lag between daily radiance and sea surface temperatures (Fig. 4). This seasonal lag between the two abiotic factors either result in

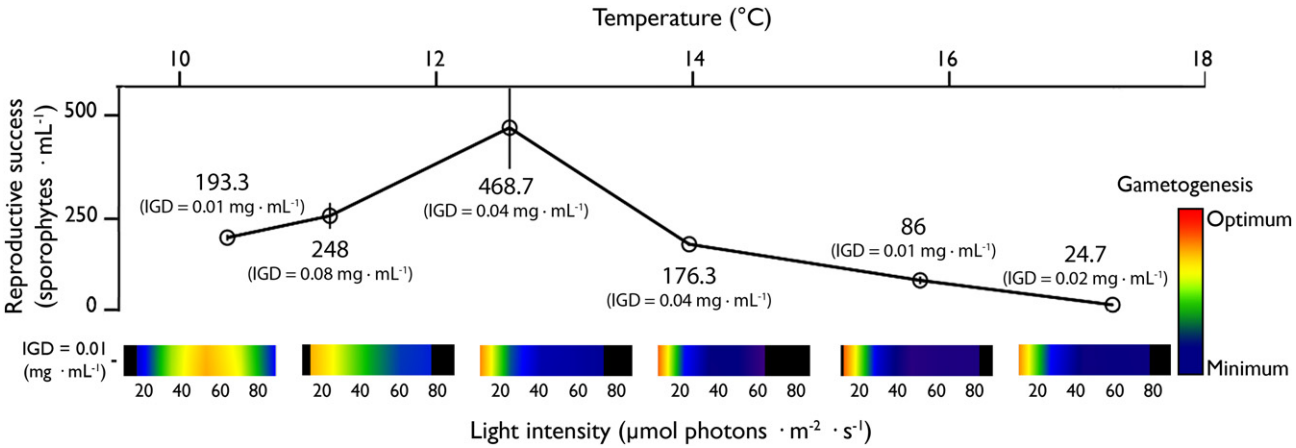

FIG. 1. The interactive influences of light intensity (µmol photons · m^{-2} · s^{-1}) and temperature (°C) on the reproductive success (sporophytes · mL^{-1}) of multiannual delayed gametophytes of *Saccharina latissima*. Figure 1A shows the optimal reproductive success of *S. latissima* gametophytes at the different temperatures (top *x*-axis). The corresponding initial gametophyte density values were added next to the points, since optimal reproduction was found at different initial gametophyte densities. Figure 1B contains smoothed contour plots that visualize how light intensity influences the reproductive success in cultures placed at an initial gametophyte density of 0.01 mg · mL^{-1} at the same six temperatures shown in Fig. 1A. Error bars represent ±SD, $n = 3$.

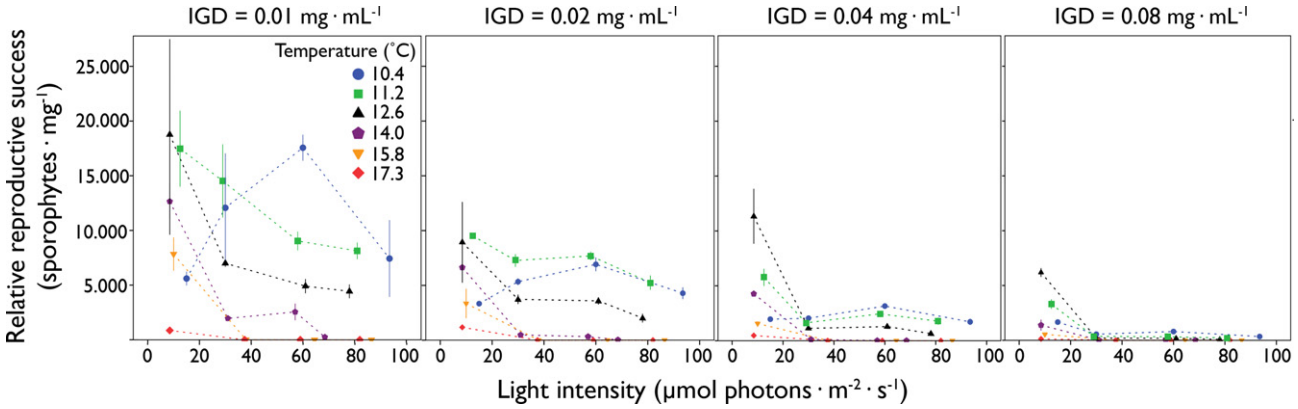

FIG. 2. The influence of light intensity and temperature on the relative reproductive success of multiannual delayed *Saccharina latissima* gametophytes. The four graphs represent the four initial gametophyte densities used in the experiment, with the relative reproductive success depicted as mean ± SD, $n = 3$. [Color figure can be viewed at wileyonlinelibrary.com]

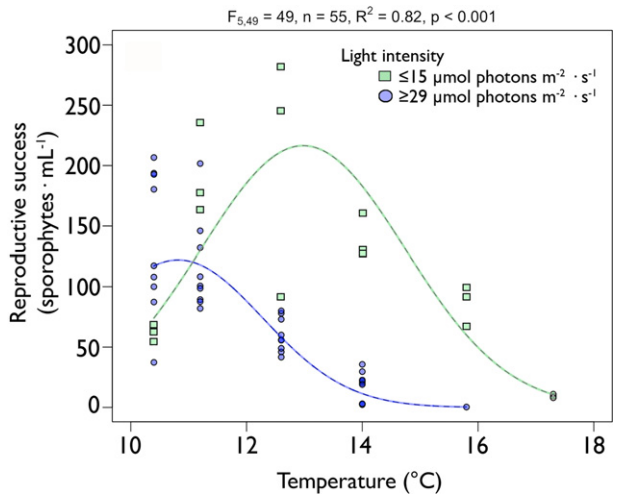

FIG. 3. The in vitro interactive effect between light intensity and temperature on multiannual delayed *Saccharina latissima* gametophyte reproduction. Values are expressed in a scatterplot, and two regressions were fitted through the data points of both low (≤15 µmol photons · m^{-2} · s^{-1}) and high (≥29 µmol photons · m^{-2} · s^{-1}) light intensities using ANCOVA. Both regressions (○ & □) are color- and symbol-coded to match to the periods in the year with the highest disparity between mean solar radiation and sea surface temperatures, to show alignment with the seasonal lag hypothesis (Fig. 4). [Color figure can be viewed at wileyonlinelibrary.com]

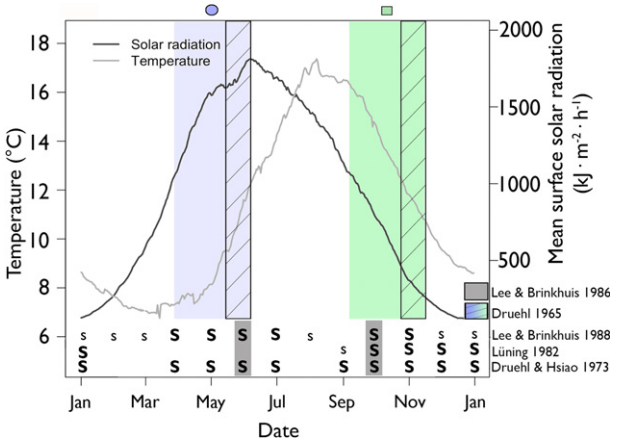

FIG. 4. Representation of the seasonal lag between mean surface solar radiation and sea surface temperatures around Flekkefjord, Norway. In vivo proxies for higher reproduction of *Saccharina latissima* gametophytes are also shown. Proxies include presumed peak zoospore releases (dashed bars) and observed peak sorus abundances (described below the graph). The abundance of sori is displayed visually with (**S**) representing ripe or abundant sorus material, (s) representing scarce sorus material, and blank spaces representing the absence of sorus material. Peak percentages of sporogenous *S. latissima* plants, observed by Lee and Brinkhuis (1986), are highlighted in gray. [Color figure can be viewed at wileyonlinelibrary.com]

(i) lower temperatures in combination with higher light intensities during spring (○) or (ii) higher temperatures in combination with lower light intensities during autumn (□). These two scenarios roughly resemble the two separate temperature optima found for the reproductive success of multiannual delayed *Saccharina latissima* gametophytes as identified under high and low light intensities, respectively (Fig. 3).

Vegetative growth. Multiannual delayed gametophytes increased vegetatively in all cultures under all experimental conditions, including at the initial gametophyte density of 0.01 mg · mL^{-1} depicted here (Fig. 5). We only show results for the lowest initial gametophyte density here, since higher initial gametophyte densities had a strong negative interactive effect on reproduction, thereby masking the relative influence of light and temperature on reproduction. Gametophyte density at this initial gametophyte density increased with increasing light intensity at all temperatures. The increase in gametophyte density started to plateau at ≥38 µmol photons · m^{-2} · s^{-1}, and the slowest overall growth was observed in cultures placed at a temperature of 10.4°C and at 15 µmol photons · m^{-2} · s^{-1}. Note that at 10.4°C, gametophyte density was still positively correlating with light intensity.

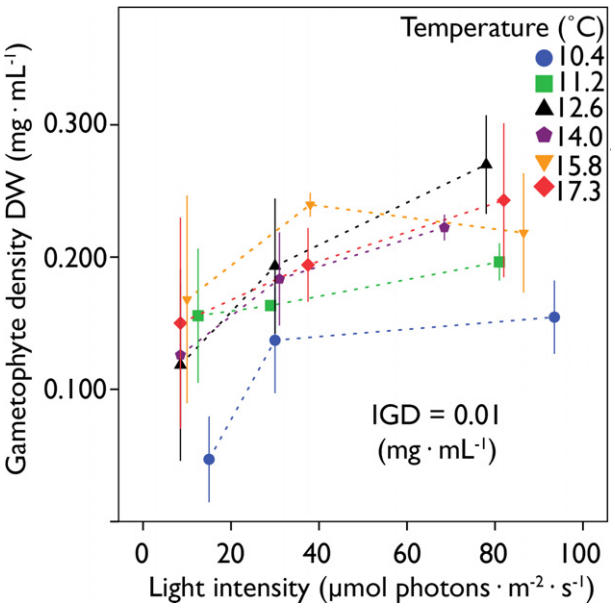

FIG. 5. Multiannual delayed *Saccharina latissima* gametophyte density (mg DW · mL^{-1}) on day 28 under different light intensities at different temperatures with the dotted lines representing linear interpolations between the different data points. Cultures started with an initial gametophyte density of 0.01 (mg DW · mL^{-1}). Values are expressed as mean ± SD, $n = 3$. [Color figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

In nature, kelp gametophytes can remain vegetative for years (Edwards 2000, Barrento et al. 2016), thereby passing multiple reproductive seasons (Hsiao and Druehl 1973) before initiating gametogenesis. In this study, we demonstrate that multiannual delayed *Saccharina latissima* gametophytes can successfully become fertilized after more than a year of vegetative growth. We furthermore show that light intensity and temperature interact to control reproductive behavior in multiannual delayed *S. latissima* gametophytes, which we suggest is most likely related to the lag between seasonal light and temperature cycles. As a result, multiannual delayed gametophytes reach optimal reproduction when the disparity between light intensity and temperature is high, such as found in spring and autumn.

The seasonal lag hypothesis. The influence of light and temperature as seasonal cues has already been well established for many autotrophs (Andrés and Coupland 2012, Singh and Singh 2015). The role of the seasonal lag between light and temperature as a life cycle control, however, has been understudied for most autotrophic organisms, including terrestrial plants (Kudoh 2019). Our experimental results on the combined effects of light, temperature, and initial gametophyte density on reproduction of multiannual delayed *Saccharina latissima* gametophytes may well be explained by the seasonal lag between daily radiation and sea surface temperatures observed around the world (Donohoe 2020). The reproductive success of multiannual delayed *S. latissima* gametophytes in our experiment increased when the disparity between temperature and light intensity increased. Multiannual delayed gametophytes appear to use the increasing disparity between solar radiation and sea surface temperature to reliably assess when spring and autumn starts, aligning their reproductive cycles with surrounding adult kelp that also might use this seasonal lag to initiate sporogenesis. If this complex response is there, it most likely uses an endogenous annual clock or circannual oscillator to synchronize with the seasons (Lüning and Dieck 1989). In evolutionary terms, the presence of an endogenous annual clock might be of fundamental importance, especially for a species that has spatiotemporally separate reproductive phases in their life cycle (Krueger-Hadfield 2020).

Alternatively, our experimental findings could be explained by the phenomenon of vegetative growth of gametophytes increasing under higher light intensities (≥29 µmol photons · m^{-2} · s^{-1}) and temperatures (≥12.6°C), with this increase in vegetative growth translating into reduced reproductive potential. However, this does not explain all the results: For at 10.4°C, the vegetative growth still correlates positively with increasing light intensities, while the reproductive success also correlates positively with increasing light intensities (Fig. 5). This indicates that vegetative growth and reproduction are not absolute antagonists, asking for a different hypothesis to complement or even override this explanation. Hence, we considered this alternative explanation less appropriate and henceforth continue to delve deeper into the seasonal lag hypothesis using multiannual delayed gametophytes.

Multiannual delayed gametophytes have been observed in vivo in similar brown seaweeds with a kelp like life history (*Desmarestia ligulata*; Edwards 2000) and *Macrocystis pyrifera* forests have been found to have mixed-parent origins, suggesting periods of longer periods of reproductive dormancy (Carney et al. 2013). However, quantifying in vivo gametophyte reproduction remains notoriously difficult (Carney and Edwards 2006), making the use of proxies for gametogenesis necessary for further validation of the seasonal lag hypothesis in multiannual delayed gametophytes. In vivo juvenile sporophyte observations, indicating recent synchronized gametogenesis events, suggest that there are multiple reproductive peaks between autumn and spring for *Saccharina latissima,* with a decrease in reproduction observed in midwinter (Parke 1948, Druehl 1965, Hsiao and Druehl 1973). Hsiao and Druehl (1973) even described seasonal events that are consistent with our described seasonal lag hypothesis, with two peaks in new macroscopic sporophyte production closely matching the periods in which increased gametophyte reproduction is predicted by our hypothesis. Furthermore, Nagasato et al. (2019)

successfully quantified in vivo *Saccharina japonica* zoospore releases for the first time and found that over a period of 2 years, clear peaks occurred in October/November with undulations and slight increases above baseline of zoospore concentrations during spring in both years, supporting the seasonal lag hypothesis. The possibility of biannual fertilization events is also supported by the fact that spores and the microscopic forms of many macroalgal species have strong tolerances to long periods of darkness (Santelices et al. 2002), suitable to survive winter conditions. We hypothesize that the increasing light conditions, accompanied with low water temperatures during spring, help delayed *S. latissima* gametophytes to synchronize gametogenesis with surrounding zoospore releases.

Another proxy for gametogenesis is the presence of sorus patches as an indicator of sporogenesis (Alsuwaiyan et al. 2019) and thus, also for recently sporulated and newly formed gametophytes. Multiannual delayed gametophytes should in this case, hypothetically, synchronize their reproduction with peak occurrences of viable newly formed gametophytes. Thanks to thorough research by Bartsch et al. (2008), three temporal scenarios can be distinguished when analyzing the observed occurrence of in vivo *Saccharina latissima* sorus patches. In the first scenario, *S. latissima* sorus patches were observable throughout the year (Parke 1948), with peak percentages of sporogenous plants observed in October and June (Lee and Brinkhuis 1986). In the second scenario, sporophytes started to develop sorus patches in autumn, which gradually disappeared starting in spring (Harries 1932, Sears and Wilce 1975, Lüning et al. 1982). Last, sorus bands were observed during two distinct periods, divided by a mid-summer and late winter hiatus when sorus patches were practically absent (Druehl and Hsiao 1977, Lee and Brinkhuis 1988). Multiannual delayed gametophytes might successfully synchronize their reproduction using the seasonal lag between solar radiation and sea surface temperature in all the above scenarios. The last scenario most closely matches our seasonal lag hypothesis with peaks of sorus patches occurring when the disparity between light intensity and temperature is greatest. We interpret this as supporting our seasonal lag hypothesis, but both in vitro and in vivo information is needed on the influence of daylength and relative temperature changes on multiannual delayed gametophyte reproduction for further validation. Moreover, the need to quantify the microscopic components of the kelp life cycles in their natural environment is apparent since the artificial environment of multiannual delayed gametophytes in laboratory studies can influence their behavior in ways that are not yet understood. This is why it is important that novel and improved molecular tools become available in the future, enabling us to further characterize these hidden components of in vivo algal communities (Hoffman and Santelices 1991, Edwards 2000, Schoenrock et al. 2020).

How light and temperature influence multiannual delayed gametophyte reproduction. Gametophyte reproduction was observable for the entire spectrum of light intensity and temperature, in accordance with the broad spectrums observed in previous studies using newly formed gametophytes. Successful reproduction was achieved using broad light intensity spectrums of white light in Lüning (1980; 5–40 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) and Lee and Brinkhuis (1988; 6–120 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}). Similarly, broad temperature ranges that resulted in reproduction were also observed in the same studies: Lüning (1980; 5–15°C, Helgoland) and Lee and Brinkhuis. (1988; 7–14°C, Long island). Such results have not only been observed in *Saccharina latissima* but also in other Laminariaceae, including *Laminaria ochroleuca* (12–18°C; Izquierdo et al. 2002), *Laminaria digitata* (5–15°C; Martins et al. 2017), and *Undaria pinnatifida* (10–15°C; Morita et al. 2003). This indicates that reproduction in the Laminariaceae is possible during multiple times of the year, as previously observed (Parke 1948, Hsiao and Druehl 1973).

Despite the similarities in the broadness of effective spectrums of light intensity and temperature on gametophyte reproduction, large discrepancies have been observed on the interactive effects of light intensity and temperature in these same studies. Lüning (1980) described a necessary increase in photon flux density in order to induce gametophyte reproduction at high temperatures (18°C), while Lee and Brinkhuis (1988) found no reproductive success at all using temperatures higher than 14°C, regardless of the applied light intensity. When addressing other members of the Laminariaceae, Izquierdo et al. (2002) found no reproductive success in *Laminaria ochroleuca* at 10°C, regardless of light intensity, while Deysher and Dean (1986) hardly observed any gametophyte reproduction in *Macrocystis pyrifera* under low light conditions ($\geq$6.7 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), regardless of temperature. The novel insights described here add on the one hand to the already large discrepancies observed in literature described above. On the other hand, they fit perfectly well in our seasonal lag hypothesis, ultimately highlighting the need to delve deeper in lesser understood (a)biotic life cycle controls governing delayed gametophyte reproduction.

Biomass density (Ebbing et al. 2020) or in this study culture age could influence the reproduction of multiannual delayed gametophyte cultures in more profound ways than it would influence the reproduction of newly formed gametophyte cultures. This is because the prolonged periods of vegetative growth not only increase the age and biomass density of multiannual delayed gametophyte cultures, but may also change genetic diversity and sex ratio of the cultures if genotypes and sexes

have different vegetative grow rates (e.g., see Destombe and Oppliger 2011). This type of selection toward individuals with higher growth rates is well documented for microalgae cultures, causing changes in the frequencies of genotypes and thus the evolution of a culture (Lakeman et al. 2009). Such lesser understood factors might ultimately influence multiannual delayed gametophyte reproduction in profound ways, thereby becoming important life cycle controls that need to be understood to enable mass cultivation of kelp using the controlled reproductive method (CRM). Understanding these life cycle controls involving CRM is important for kelp domestication as it opens up fundamentally new and different ways of kelp farming and kelp breeding.

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AUTHOR CONTRIBUTIONS

A. P. J. Ebbing: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **R. Pierik:** Funding acquisition (supporting); Supervision (supporting); Validation (supporting); Writing-review & editing (equal). **G. S. Fivash:** Data curation (supporting); Software (supporting); Validation (supporting); Visualization (supporting); Writing-review & editing (equal). **N. C. J. van de Loosdrecht:** Investigation (supporting). **T. J. Bouma:** Supervision (supporting); Validation (supporting); Writing-review & editing (equal). **J. C. Kromkamp:** Supervision (supporting); Validation (supporting); Writing-review & editing (equal). **K. Timmermans:** Funding acquisition (lead); Project administration (lead); Resources (lead); Supervision (lead); Validation (supporting); Writing-review & editing (equal).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Calibration line between the chlorophyll a concentration (mg Chl·m^{-3}), and *Saccharina latissima* gametophyte dry weight per mL (mg DW·mL^{-1}). Gametophyte dry weights are extrapolations from 60 mL cultures, whose [Chl] concentration were measured using a FRRF fluorometer. The linear regression and correlation coefficient were $y = 7E - 05x - 9E - 05$ and 0.975, respectively.

Figure S2. The light absorbance spectrum of *Saccharina latissima* gametophytes (black line) projected over the spectral distribution of White L.E.D. light. Light was measured at different wavelengths from 400 nm until 700 nm, and peak emission strength was normalized to 1 and plotted against the absorbance of the culture (%).

Figure S3. Photo of the experimental setup with some of the incubators used. The LED panels were hanged in front of the see-through doors of the incubators, enlightening the gametophyte cultures with white light.

Table S1. The seven cultivation phases described by Vodouhe et al. 2012 are used to see how far we have come in the domestication process of kelp as an aquatic crop. Based on a literature study, we assessed that kelp aquaculture is hovering between the 4th and 6th cultivation phase, depending on the cultivation method used.

Table S2. Games–Howell post hoc analysis for the influence of light intensity on gametophyte reproduction at an IGD of 0.01 mg·mL^{-1}, after we found significant differences using the robust test of variance. The mean difference is significant at $P < 0.05$.

Table S3. The ANCOVA analysis of the influence of light intensity (μmol photons·m^{-2}·s^{-1}) and temperature (°C) on the reproductive success of MAD gametophytes.