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APPLIED STUDIES

Conditions for staggering and delaying outplantings of the kelps *Saccharina latissima* and *Alaria marginata* for mariculture

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Abstract

We describe a method for production of kelp using meiospore seeding creating flexibility for extended storage time prior to outplanting. One bottleneck to expansion of the kelp farming industry is the lack of flexibility in timing of seeded twine production, which is dependent on the fertility of wild sporophytes. We tested methods to slow gametophyte growth and reproduction of early life stages by manipulating temperature of the kelp *Saccharina latissima*. Reducing temperature from 12°C to 4°C reduced gametophyte size, sporophyte size, egg production, and sporophyte production and subsequently was the best candidate condition for storage experiments of seeded twine. Next, we examined how storage of *Alaria marginata* and *S. latissima* seeded twine at 4°C under differing nutrient concentrations affected the viability of sporelings after being moved into optimal growth conditions. Seeded twine storage at 4°C with no alteration to culturing media showed no negative effects in sporophyte density and sporophyte length for both species. This method for seeded twine storage, "cold banking," allowed seeded twine storage for at least an additional 36 days compared to standard methods, with a total of 56 days spent in the hatchery providing opportunity for

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outplanting timing and staggering to enhance aquaculture efficiency.

KEYWORDS
aquaculture, cold banking, hatchery, kelp, macroalgae, seaweeds

1 | INTRODUCTION

1.1 | Commercial kelp mariculture bottlenecks

A critical step in commercial kelp mariculture is the production of viable seeded twine. There are a variety of methods for the production of seeded twine including cloning cultures and harvesting reproductive material from wild fertile sporophytes. Isolated, cloned gametophyte cultures can be stored for decades and revived to form sporophytes by combining small fragments of the male and female cultures (Barrento, Camus, Sousa-Pinto, & Buschmann, 2016; Redmond, Green, Yarish, Kim, & Neefus, 2014). This method can produce many offspring while taking very little fertile sporophytes from wild populations, provide a continuous supply of material (Barrento et al., 2016; Redmond et al., 2014), and is used regularly in the seaweed mariculture industry in Japan, China, and South Korea (Pereira & Yarish, 2008). However, cloned cultures require complex and often costly laboratory resources and meticulous efforts to initiate and maintain (Goecke, Klemetsdal, & Ergon, 2020). Another cultivation method, called meiospore seeding uses wild fertile sporophytes to produce meiospores, which settle onto twine (hereafter "seed"), and is the focus of this study. The meiospores grow into male and female gametophytes, produce zygotes, and form sporophyte recruits, which are later strung on longlines in an ocean farm. This method is less labor intensive to initiate, does not require a state-of-the-art facility, and promotes gene conservation of wild populations in comparison to cloned cultures (Allendorf, Luikart, & Aitken, 2012; Utter & Epifanio, 2002). This method is also used in China, Japan, and South Korea (Pereira & Yarish, 2008), and the developing kelp mariculture industry across United States of America.

Using meiospore settlement methods has a major drawback for large-scale mariculture because it relies on the seasonal availability of sorus. Furthermore, when the sporophytes reach 2–3 mm, they must be outplanted as the spatial and environmental needs of the growing juvenile sporophytes cannot be met in the hatchery (Redmond et al., 2014). If the amount of time from meiospore seeding to the desired outplanting date is too long, the quality of the seeded twine will decrease as juvenile sporophytes start withering and falling off the seeded twine (Redmond et al., 2014). To reduce reliance on wild fertile sporophytes, one modified method suggests that sorus induction can be accomplished by modifying light periods with *S. latissima* (Forbord et al., 2012). However, this has not been confirmed as a reliable methodology with other kelp species in the Northeast Pacific. The discrepancy between sorus availability and optimal outplanting times may affect yield, farming logistics, and the overall economic output. This limitation led us to explore methods to store seeded twine (hereafter "seed") for extended periods for the kelps *S. latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl, and G.W. Saunders and *A. marginata* Postels and Ruprecht. A method which extends seed storage time will facilitate the ability to stagger seed twine outplantings, providing flexibility in harvest times, reducing a workload bottleneck both in the hatchery and on the ocean farm.

1.2 | Abiotic factors affecting gametophyte growth

Brown algal gametophytes are considered microscopic forms of the species that act as banks that can overwinter (Edwards, 2000) or survive during undesirable environmental conditions such as El Niño events (Carney, 2011;

Carney & Edwards, 2006; Carney, Edwards, & Nin, 2010). Previous research has highlighted that certain abiotic factors, including light, temperature, growth media, and season, may slow both gametophyte growth and reproduction and are detailed below.

1.2.1 | Light

Light is a key factor regulating gametophyte growth in *Saccharina* spp. gametophytes (Egan, Vlasto, & Yarish, 1989; Hsiao & Druehl, 1973; Lee & Brinkhuis, 1988; Lüning & Neushul, 1978; Ratcliff, Soler-Vila, Hanniffy, Johnson, & Edwards, 2017). The saturation threshold for gametophyte growth of many species is 20 μmol m^{-2} s^{-1} (Lüning & Neushul, 1978). This light range was confirmed for *S. latissima*, where a light range of 5 to 20 μmol m^{-2} s^{-1} resulted in optimal gametophyte growth (Egan et al., 1989; Lee & Brinkhuis, 1988). Light regimes in the range of 5 to 120 μmol m^{-2} s^{-1} have little influence on sporophyte germination for *S. latissima* (Lee & Brinkhuis, 1988). During the microscopic sporophyte stage, immediately following the gametophyte stage, it has been suggested that 20 to 30 μmol m^{-2} s^{-1} is the irradiance saturation threshold for growth of both *S. latissima* and *Alaria esculenta* from the North Atlantic (Han & Kain (Jones, 1996).

1.2.2 | Temperature

The optimal growing temperature for kelp in North America is widely considered to be 12°C (Redmond et al., 2014). Reducing the temperature to 4°C has been shown to slow *S. latissima* gametophyte growth in their first few weeks of life (Augyte, Yarish, & Neefus, 2019; Egan et al., 1989; Lee & Brinkhuis, 1988), suggesting that reduced temperatures may be a viable method for controlling the kelp life cycle. For example, *Macrocystis pyrifera* gametophytes from Chile cultivated in clone culture can remain viable with little care for at least 5 years by utilizing cold (<12°C) temperatures (Barrento et al., 2016). Although low temperatures have been shown to slow gametophyte growth, there have been few studies examining how holding gametophytes in low temperatures affects the commercial quality of seed when returned to optimal growing conditions.

1.2.3 | Media

Media composition and concentration is well known to effect kelp phenology and growth in all life stages (Kerrison et al., 2016; Lewis, Green, & Afzal, 2013; Stekoll, Peeples, & Raymond, 2021; Suzuki, Kuma, & Matsunaga, 1994). Iron deficient culture media halts gametogenesis in female gametophytes of many kelp species but does not stop gametophyte filamentous growth (Lewis et al., 2013; Stekoll et al., 2021; Suzuki et al., 1994). Although the use of iron deficient media is potentially useful for commercial production of seed, how storage time in iron deficient media affects future phenology of kelp when moved into optimal growing conditions is not well understood. Therefore, in our experience, it is necessary to identify additional factors such as nutrient concentration and composition that slow both gametophyte growth and delay reproduction.

1.2.4 | Seasonal effects

In addition to abiotic factors affecting microscopic phenology, the season of meiospore release can contribute to variability in gametophyte growth and reproduction (Egan et al., 1989; Lee & Brinkhuis, 1988; Mohring, Kendrick, Wernberg, Rule, & Vanderklift, 2013; Nielsen, Kumar, Soler-Vila, Johnson, &

Bruhn, 2016). In one study, meiospores of *S. latissima* originating from sorus in February, late in the fertile season, grew slower than those originating in April, at the start of the fertile season (Nielsen et al., 2016). Given these patterns, understanding how seasons affects microscopic phenology of gametophytes is an important element in understanding the ecological strategies employed and, therefore, the natural meiospore seeding variability to be expected by the mariculture industry.

1.3 | Aims

Here, we identify environmental conditions and methods to hold *S. latissima* and *A. marginata* seeded twine in a hatchery setting for an extended culturing period by manipulating irradiance, nutrient concentrations, presence and absence of iron, and temperature. We hypothesized that temperature would be the most useful variable for seed storage as previous studies have described relatively dramatic responses to temperature changes compared to other factors such as light and nutrients.

2 | METHODS

We split our study into two sets of experiments. First, experiments were conducted on *S. latissima* (2.2) by examining how light, temperature and season-affected gametophyte growth, and reproduction. Next, we applied results from Experiment 2.2 to a seed storage experiment (2.3) which examined how storage in conditions that stall gametophyte growth and reproduction affect the viability of the seed when outplanted. We conducted this experiment (2.3) on both *S. latissima* and *A. marginata* to test if the factors we selected from 2.2 could be applied to another commercially grown kelp species.

2.1 | Meiospore seeding

We designed our experiments to mirror the commercial mariculture process of meiospore seeding. After sporophytes were collected in the field, sori were cleaned of epiphytes, treated with a 2 mL/L Betadine® solution, washed with sterile seawater (SSW), and then dried and stored overnight in a dark incubator at 4°C on paper towels lightly moistened with SSW. After 16 hr (Day 0 of each experiment) meiospores were released by placing dried sori in individual sterile beakers with SSW at 12°C under light irradiance of 30 to 60 μmol m^{-2} s^{-1}. After quantifying motile meiospore density with a hemocytometer, the meiospore solution was distributed into experimental units in the form of petri dishes or seeded twine pipe replicates. Experimental replicates were placed randomly in an incubator at 12°C and left overnight (16 hr) while meiospores settled. The next day, experimental replicates were moved to the different environmental treatments depending on the experiment. For the duration of the experiments, we rotated the experimental replicate locations and orientations in incubators.

Incubators were set at 12:12, L:D photoperiod using cool-white fluorescent lights with a color rendering index range of 89 to 90 and a color temperature of 4,100 K. We made weekly measurements of irradiance using a LI-COR QUANTUM photometer. We added Provasoli's enriched seawater (Provasoli, 1968) with added iodine (PES) stock solution in concentrations depending on the experimental treatment detailed below. The PES was modified by the addition of 1.4 mg/L iodine to the stock solution (Tatewaki, 1966) and by excluding any vitamins and any buffer. Iron was omitted in the iron-deficient media. During weekly media changes, 2 mL of 0.25 g/L germanium dioxide were added to each liter of culture medium to prevent diatom contamination.

2.2 | Effect of temperature, irradiance, and meiospore release month on gametophyte growth and reproduction

The effects of temperature, irradiance, and meiospore release month (season) on microscopic growth and reproduction of *S. latissima* were investigated under three different temperatures 4°C, 8°C, and 12°C, two irradiance regimes <10 and 30–50 μmol m^{-2} s^{-1}, using a 12:12, L:D photoperiod, and two different meiospores release dates (July 5 and August 13, 2018).

For Experiment 2.2, we collected fertile *S. latissima* from Auke Bay Recreation Area (58.375 N, 134.730 W) on July 4 and August 12, 2018, one day before each meiospore release date. Sori from a mixture of three to six thalli were used for each meiospore release. A solution of 10 mL of 1,000 meiospores/mL was pipetted into each petri dish replicate. We first tested for an incubator effect through a control experiment outlined in Appendix A. This experiment found no difference in gametophyte length among incubators ($F_{4,80} = 1.310$, $p = .274$) after 14 days (Figure A1; Table A1). For Experiment 2.2, nine replicate petri dishes were used for each treatment. On day one, petri dishes were randomly assigned to two irradiance and three temperature treatments and cultured for 4 weeks. A temperature logger (Onset HOBO pendant) was placed in each incubator to track temperature (Figure B3). Experiments from both seeding dates ran 32 days with sampling starting at Day 28. Gametophyte and sporophyte measurements were quantified with a LEICA DMC 2900 camera attached to a LEICA DMi8 microscope. We randomly sampled gametophyte and sporophyte size by taking measurements of 30 individuals from each petri dish. The longest gametophyte diameter was taken by photo cataloguing and measured with ImageJ (v1.51s). Sporophyte lengths were measured under a microscope using a micrometer. The fraction of female gametophytes with eggs (FFGE) and sporophytes (FFGS) was determined for each dish as a measure of gametophyte phenology.

Three-way analyses of variance (ANOVA) were used to evaluate the effect of irradiance, temperature, meiospores release month, and incubator nested within temperature on gametophyte length, sporophyte length, and FFGE and FFGS. Gametophyte and sporophyte lengths were square root transformed and FFGE was arcsine-square root transformed. Model selection was carried out using backward selection, selecting the best fit model with the lowest value by 2 AIC points (Burnham & Anderson, 2004; Burnham, Anderson, & Huyvaert, 2011) using the stepAIC function in R (v3.5.1) package MASS (Venables, Bates, Hornik, Gebhardt, & Firth, 2002). Statistically similar groups were determined by Tukey HSD tests in package agricolae (Mendiburu, 2017).

2.3 | Effect of hatchery storage methods on seeded twine quality

Experiment 2.2 identified low temperatures of ~4°C as a key storage condition across multiple response variables (see details in the Results section). Following this result, we examined, for *A. marginata* and *S. latissima*, how seed storage at 4°C could affect the quality and viability of the kelps after they are returned to optimal growing conditions at 12°C. This experiment had four stages, the "seeding stage" to "storage stage" to "growth stage" and finally to the "ocean tank stage" (Figure 1). This experiment addressed how storage in different concentrations of nutrients and the presence or absence of iron in the media (Table 1) affected the quality and viability of the sporophytes after storage. We quantified seed quality as measured by sporophyte density and length at the end of the growth and ocean tank stages to examine how storage treatment affected the long-term viability of the seed after returning to conditions of optimal growth (Figure 1).

All treatments contained four replicates consisting of a 5 cm long, 4 cm diameter PVC pipe wrapped with 2 m of *Kuremona* twine cultured in a 250 mL beaker. Control (PControl) replicates went through all the experimental stages except the storage stage (Figure 1). Experimental treatments consisted of varying PES concentrations with the presence or absence of iron (Table 1). The *A. marginata* trial consisted of meiospore release on June 6, 2019 from three sporophytes collected from Auke Bay Recreation Area the day before. The *S. latissima* trial consisted of meiospore release on July 16, 2019 from five sporophytes collected from Tee Harbor (58.431 N, 134.764 W) the day before.

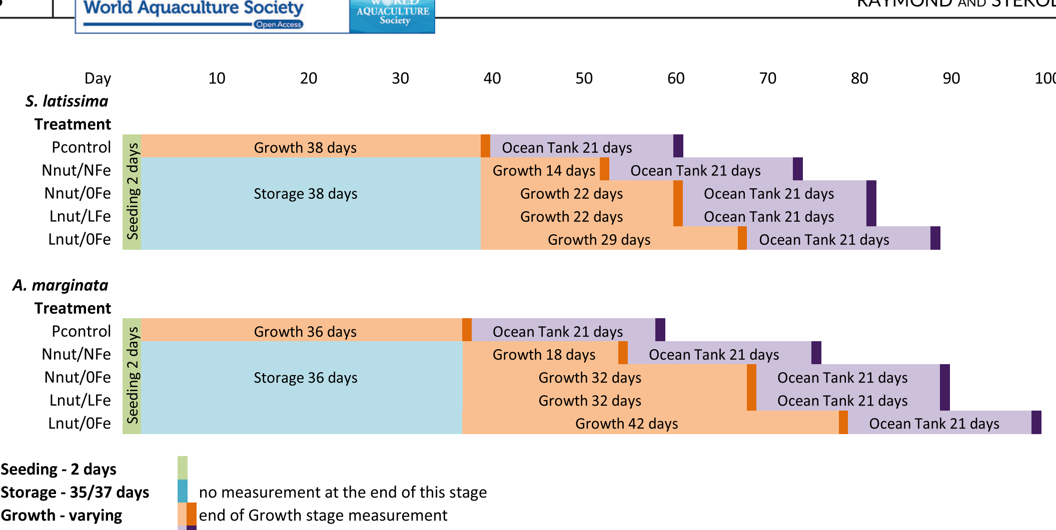

FIGURE 1 Experimental design testing the effects of hatchery storage methods on seed quality. Pcontrol, no storage stage. Storage stage: Nnut/NFe, normal ½ PES with normal iron; Nnut/0Fe, normal ½ PES with no added iron; Lnut/LFe, 1/20 PES with 1/10 iron; Lnut/0Fe, 1/20 PES with no added iron. The Storage stage was 4°C, 12L:12D at 33 to 44 μmol m^{-2} s^{-1}. Growth stage: normal ½ PES with normal iron, same light conditions as the storage stage but at 12°C. Ocean tank stage: filtered seawater with no added nutrients, ambient light at 54 and 80 μmol m^{-2} s^{-1} with an 16L:8D photoperiod

TABLE 1 Treatment conditions in the storage stage and growth stage for Experiment 2.3. Experiment was conducted once with *Alaria marginata* and once with *Saccharina latissima*. ½ PES was used as the base concentration. NA, not applicable; NNut, normal nutrients; LNut, low nutrients; NFe, normal iron levels; LFe, low iron levels; 0Fe, no added iron. The storage stage started 2 days after meiospores release and lasted 35 days for *A. marginata* and 37 days for *S. latissima*

	Storage stage				Growth stage		
Storage treatment	Temp (°C)	Fraction of added nutrient (PES)	Fraction of added iron (PES)	Added iron conc. (mg/L)	Temp (°C)	Fraction of added nutrient (PES)	Fraction of added iron (PES)
PControl	NA	NA	NA	NA	12	1/2 PES	1
NNut/NFe	4	1/2 PES	1	2.27	12	1/2 PES	1
LNut/LFe	4	1/20 PES	1/10	0.27	12	1/2 PES	1
NNut/0Fe	4	1/2 PES	0	0	12	1/2 PES	1
LNut/0Fe	4	1/20 PES	0	0	12	1/2 PES	1

During the seeding, storage, and growth stages, light conditions ranged between 33 and 44 μmol m^{-2} s^{-1}. Seawater samples were taken throughout the duration of the experiment to monitor dissolved nutrients. Seawater nitrate ranged between 10.74 and 26.53 μM and phosphate concentrations ranged between 1.48 and 2.91 μM in the natural seawater. All treatments experienced 12:12, L:D photoperiod after meiospore settlement.

After 2 days in seeding conditions (at 12°C), replicates were moved into their storage stage at 4°C with four different nutrient treatments: (a) a standard concentration of PES commonly used in kelp culturing (½ PES) with full Fe addition (NNut/NFe); (b) 1/20th PES, 1/10th iron addition (LNut/LFe); (c) ½ PES, no Fe addition (NNut/0Fe); and (d) 1/20th PES, no Fe addition (LNut/0Fe) (Table 1). The controls (PControl) were left in 12°C with ½ strength PES plus iron and moved directly to the growth stage. The storage stage at 4°C lasted 35 days for *A. marginata* and

37 days for *S. latissima*. The subsequent growth stage was designed to promote sporophyte growth and had a culturing environment of 12°C with ½ strength PES plus iron (Table 1; Figure 1). Because the nutrient treatments in the storage stage promoted different growth and phenological characteristics, the length of time each treatment remained in the growth stage differed among treatments. We termed this time the "sporophyte grow out time" (SGOT) or the number of days it takes to grow sporophytes to an approximate length of 1–2 mm. SGOT was determined visually for each treatment group. On the last day of the growth stage, the quality of the growing plants was determined by quantifying sporophyte length and sporophyte density using a LEICA DMi8 microscope. Sporophyte length and density consisted of an average of nine measurements for each pipe replicate.

At the end of the growth stage, replicates were outplanted in one "ocean tank," consisting of a rectangular 260 L surge tank (61 × 214 × 20 cm) (Stekoll & Else, 1992) with a recirculating chiller programed to maintain temperatures between 9°C and 11°C. Filtered seawater (1 μ) continuously flowed into the tank at a rate of 4.7 L/min, with a residence time of approximately 55 min. During the ocean tank stage, irradiance ranged between 54 and 80 μmol m^{-2} s^{-1} with an 8:16 L:D photoperiod. From each pipe replicate, the seeded twine was cut into three 7.6 cm segments and attached with zip-ties to a weighted polypropylene line. This gave a density of one twine segment per 4.4 L. After 3 weeks, we measured sporophyte length and sporophyte density on each replicate. Sporophyte length was quantified by measuring the nine largest sporophytes for each replicate. Sporophyte density for each replicate was measured under a magnifying glass (5×) by counting the number of sporophytes on a 2.0 cm section of each seeded twine segment.

Results were evaluated with a one-way ANOVA for each species with storage method as the factor of interest and statistically similar groups were determined by Tukey HSD. Sporophyte density of *A. marginata* at the end of the ocean tank stage was square root transformed to satisfy normality assumptions. Sporophyte density of *S. latissima* on outplant day was square root transformed.

3 | RESULTS

3.1 | Effect of temperature, irradiance, and seed date on growth and reproduction

Temperature had a strong effect on *S. latissima* gametophyte length ($F_{2,165} = 68.297$, $p < 0.001$), as well as meiospore release month ($F_{1,165} = 48.088$, $p < 0.001$) (Table 2). Overall, gametophyte length was lowest from plants collected in the month of August. The lowest growth was observed at 4°C from both months and at 8°C in August (Figure 2). Across irradiance treatments, the average gametophyte length at 4°C was 33.5% of the average length at 12°C for the July meiospore release and as low as 26.6% of the length at 12°C for the August meiospore release (Figure 2). Within the 8°C and 12°C temperature treatments, average gametophyte length from the August meiospore release was 52.8% of the length of gametophytes from July (Figure 2). Within the 8°C temperature treatment, gametophytes from the August date were 44.2% the length of July gametophytes (Figure 2).

Temperature also showed a strong effect on *S. latissima* sporophyte length ($F_{2,118} = 49.258$, $p < 0.001$). Meiospore release month ($F_{1,118} = 34.944$, $p < 0.001$) and irradiance ($F_{1,118} = 12.812$, $p = 0.001$) also effected sporophyte length (Table 2). Unlike the growth of the gametophytes, sporophyte length was lowest overall in July. The lowest average length was observed at 4°C in both months. Sporophyte length was highest in August at 12°C (Figure 2). Across irradiance treatments, the length of sporophytes at 4°C was 33.5% of the length at 12°C for July and as low as 26.6% of the length at 12°C for August (Figure 2). Within the 12°C temperature treatment, the July-sporophyte length was 47.2% of the average sporophyte length in August (Figure 2).

S. latissima reproductive success in terms of FFGE was affected by temperature ($F_{2,161} = 110.860$, $p < 0.001$), meiospore release month ($F_{1,161} = 5.588$, $p = 0.019$), and irradiance ($F_{1,161} = 6.358$, $p = .013$) (Figure 3; Table 2). We found some indication of an incubator effect ($F_{4,161} = 2.391$, $p = 0.053$) on FFGE, further examined in Appendix B. Overall, gametophyte fertility expressed as FFGE was highest from the July meiospores release at 12°C. The lowest FFGE was observed at 4°C from both months. FFGE at 4°C was as low as 2.4 and 7.1% of what the FFGE was at

TABLE 2 Final model selection results of three-way ANOVAs on the effects of temperature, light treatment and meiospore release month on gametophyte length, sporophyte length, fraction of female gametophytes with eggs (FFGE), and fraction of female gametophytes with sporophytes (FFGS) (Experiment 2.2)

	df	SS	MS	*F* value	*p*
Gametophyte length (model 2, AIC = −888)					
Irradiance	1	0.009	0.009	1.381	0.242
Meiospore release month	1	0.313	0.313	48.088	<0.001
Temperature	2	0.888	0.444	68.297	<0.001
Irradiance: meiospore release month	1	0.008	0.008	1.195	0.276
Irradiance: temperature	2	0.016	0.008	1.230	0.295
Meiospore release month: temperature	2	0.007	0.003	0.527	0.591
Incubator nested in temperature	4	0.044	0.011	1.685	0.156
Residuals	165	1.073	0.007		
Sporophyte length (model 2, AIC = 349.5)					
Meiospore release month	1	446.510	446.510	34.944	<0.001
Temperature	2	1,258.799	629.400	49.258	<0.001
Irradiance	1	163.707	163.707	12.812	0.001
Meiospore release month: temperature	2	160.210	80.105	6.269	0.003
Irradiance: meiospore release month	1	7.051	7.051	0.552	0.459
Irradiance: temperature	2	246.295	123.148	9.638	<0.001
Incubator nested in temperature	4	190.132	47.533	3.720	0.007
Residuals	118	1,507.770	12.778		
FFGE (model 1, AIC = −494.77)					
Irradiance	1	0.356	0.356	6.358	0.013
Meiospore release month	1	0.313	0.313	5.588	0.019
Temperature	2	12.429	6.215	110.860	<0.001
Irradiance: meiospore release month	1	0.032	0.032	0.571	0.451
Irradiance: temperature	2	0.239	0.120	2.135	0.122
Meiospore release month: temperature	2	1.407	0.704	12.552	<0.001
Incubator nested in temperature	4	0.536	0.134	2.391	0.053
Irradiance: meiospore release month: temperature	2	0.113	0.056	1.005	0.368
Residuals	161	9.025	0.056		
FFGS (model 4, AIC = −550.94)					
Irradiance	1	0.402	0.402	9.438	0.002
Meiospore release month	1	0.234	0.234	5.506	0.020
Temperature	2	3.559	1.780	41.811	<0.001
Irradiance: meiospore release month	1	0.005	0.005	0.123	0.726
Meiospore release month: temperature	2	0.191	0.096	2.248	0.109
Residuals	169	7.193	0.043		

12°C for the July and August meiospores release, respectively (Figure 3). Within the 12°C temperature treatment, the FFGE for the August date was 39.6% of the average FFGE of July (Figure 3).

The results for the appearance of sporophytes were similar to those for egg formation. We found significant effects on the FFGS for temperature ($F_{2,169} = 41.811$, $p < 0.001$), meiospore release month ($F_{1,169} = 5.506$,

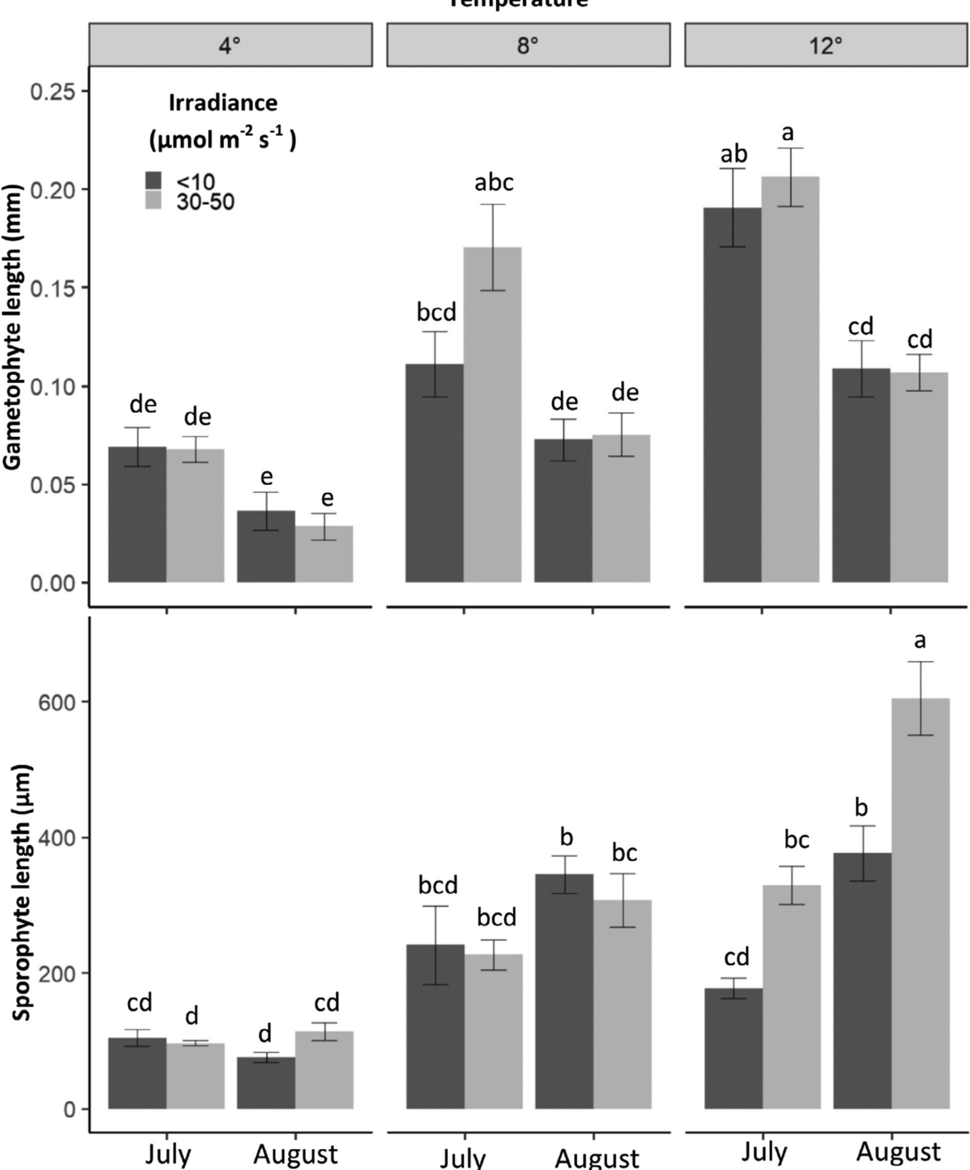

FIGURE 2 *Saccharina latissima* gametophyte length (top) and sporophyte length (bottom) (Experiment 2.2) as a function of irradiance and temperature treatments on two meiospore release months. Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error. There were nine replicates in each combination of light, temperature and meiospores release month

$p = 0.020$) and irradiance ($F_{1,169} = 9.438$, $p = 0.002$) (Table 2). Although we found an overall effect of meiospore release month and light treatment on FFGS, these results are not confirmed by post hoc tests within treatments of temperature and meiospore release month (Figure 3). Overall, FFGS was highest in the month of July at 12°C. The lowest FFGS was observed at 4°C in July. The FFGS at 4°C was 15.7% of what the FFGS value was at 12°C for July across irradiance treatments (Figure 3). The FFGS in August at 4°C was 40.2% of what the FFGS value was at 12°C across irradiance treatments (Figure 3).

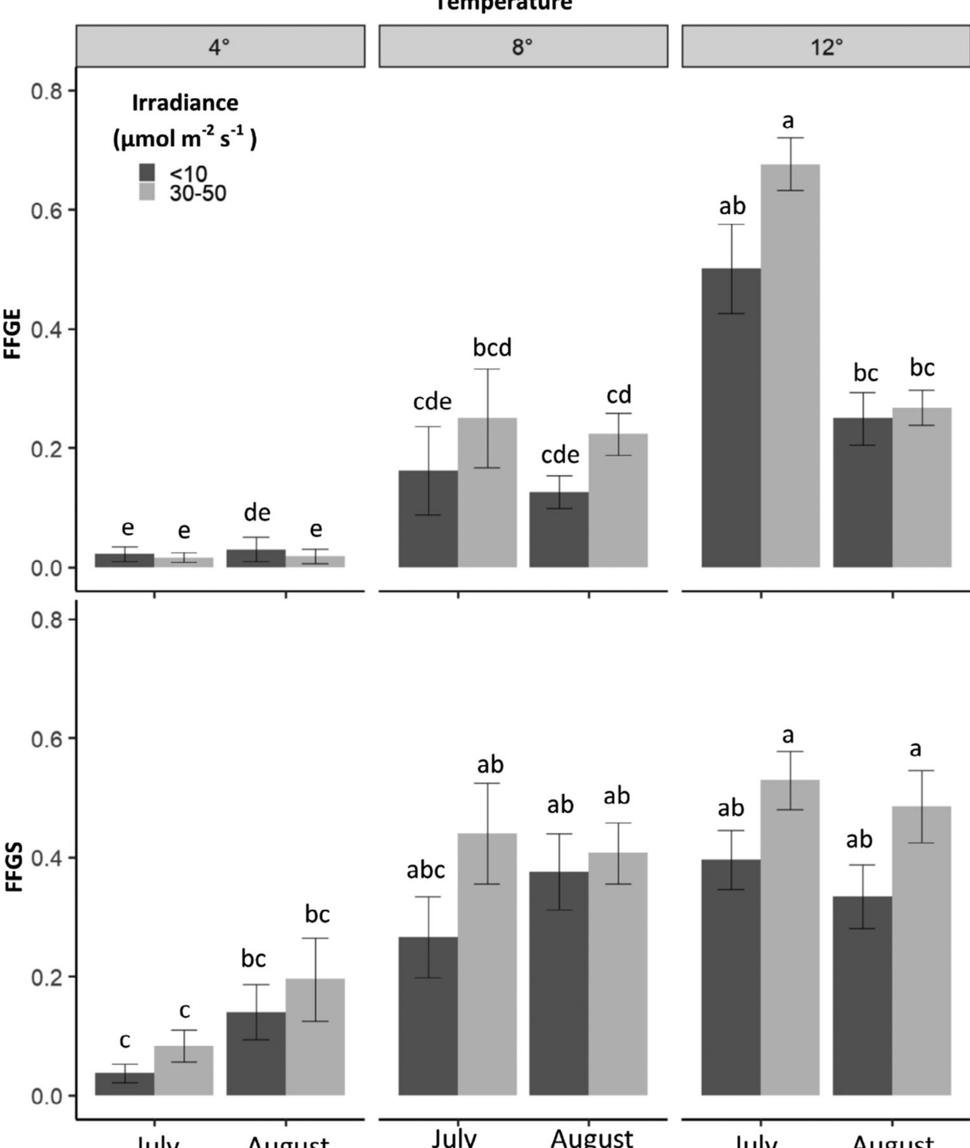

FIGURE 3 *Saccharina latissima* gametophyte reproductive success (Experiment 2.2) as a function of different irradiance and temperature treatments on two meiospore release months. Top: FFGE (fraction of female gametophytes with eggs). Bottom: FFGS (fraction of female gametophytes with sporophytes). Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error. There were nine replicates in each combination of light, temperature and meiospores release month

3.2 | The effects of hatchery storage methods on seed quality

3.2.1 | *Alaria marginata*

In this experiment which examined the effect of storage treatment on seed quality, the SGOT of *A. marginata* varied among storage treatments with the storage treatment at 4°C with normal nutrients (NNut/NFe) being the shortest

TABLE 3 Sporophyte grow out time (SGOT) for each treatment of each species (Experiment 2.3). SGOT represent the number of days spent in the growth stage or the time from the end of the storage stage to when sporophytes reached an approximate length of ~1 to 2 mm. Refer to Table 1 for treatment conditions

Sporophyte grow out time (days)		
	Species	
Treatment	*Alaria marginata*	*Saccharina latissima*
PControl	36	38
NNut/NFe	18	14
LNut/LFe	32	22
NNut/0Fe	32	22
LNut/0Fe	42	29

at 18 days (Table 3). Replicates from treatments LNut/LFe, NNut/0Fe, and LNut/0Fe had heavy gametophyte growth and only inconsistent sporophyte growth at the end of the growth stage (Figure S1, S2). Storage treatment significantly affected *A. marginata* sporophyte density at the end of the growth stage ($F_{4,15} = 4.244$, $p = 0.017$) where NNut/NFe had greater sporophyte densities then all other treatments except for LNut/0Fe ($p < 0.05$) (Table 4; Figure 4). Storage treatment had a significant effect on *A. marginata* sporophyte length at the end of the growth stage ($F_{4,15} = 3.502$, $p = 0.033$), although the only significant difference among treatments was that the NNut/NFe treatment had longer blades than the PControl ($p < 0.05$) (Table 4; Figure 4). At the end of the ocean tank stage, we found strong evidence of an effect of storage treatment on *A. marginata* sporophyte density ($F_{4,15} = 28.883$, $p < 0.001$) with NNut/NFe having higher densities than all other treatments (Table 4; Figures 5 and 6). Storage treatment affected *A. marginata* sporophyte length ($F_{4,15} = 7.787$, $p = 0.001$); the PControl was significantly shorter than the treatments of NNut/NFe and NNut/0Fe (Figure 5).

3.2.2 | Saccharina latissima

SGOT of *S. latissima* varied among storage treatments and was similar to that for *A. marginata*. The shortest SGOT was 14 days in the NNut/NFe treatment (Table 3). Similar to *A. marginata*, replicates from treatments LNut/LFe, NNut/0Fe, and LNut/0Fe had heavy gametophyte growth and patchy sporophyte growth. The response of *S. latissima* was different than that of *A. marginata*. At end of the growth stage, *S. latissima* sporophyte density ($F_{4,15} = 0.722$, $p = 0.590$) and length ($F_{4,15} = 1.478$, $p = 0.258$) were not affected by storage treatment (Table 4; Figure 4). Similar results were confirmed at the end of the ocean tank stage where sporophyte density ($F_{4,15} = 1.562$, $p = 0.236$) and length ($F_{4,15} = 2.570$, $p = 0.081$) showed no effect of storage treatment (Table 4; Figure 5).

4 | DISCUSSION

As hypothesized, we identified temperature to be a key variable for the successful storage of *S. latissima* seed in Experiment 2.2. Reducing temperature from 12°C to 4°C significantly reduced all measurements of *S. latissima* microscopic size and reproduction including gametophyte and sporophyte length, egg production, and sporophyte production. In Experiment 2.3, storage of *S. latissima* and *A. marginata* at 4°C in standard nutrient media showed no negative effects for seed quality at the time or 3 weeks after outplanting. Combining the results of these two experiments, we introduce a hatchery methodology which can delay the progression of seed so that outplanting can be

TABLE 4 Results examining the effects of hatchery storage methods on seed quality (Experiment 2.3). A one-way ANOVA is displayed for each response variable for each species

	Alaria marginata					*Saccharina latissima*				
	df	SS	MS	*F*	*p*	*df*	SS	MS	*F*	*p*
Sporophyte density at end of optimal growth stage										
Storage treatment	4	365.040	91.260	4.244	.017	4	0.411	0.103	0.722	0.590
Residuals	15	322.550	21.503			15	2.132	0.142		
Sporophyte length at end of optimal growth stage										
Storage treatment	4	2,102,480.000	525,620.000	3.502	.033	4	2,146,638.000	536,659.000	1.478	0.258
Residuals	15	2,251,117.000	150,074.000			15	5,447,792.000	363,186.000		
Sporophyte density at end of the ocean tank stage										
Storage treatment	4	57.154	14.289	28.883	<.001	4	1.436	0.359	1.562	0.236
Residuals	15	7.421	0.495			15	3.450	0.230		
Sporophyte length at end of the ocean tank stage										
Storage treatment	4	52.825	13.206	7.787	.001	4	15.174	3.794	2.570	0.081
Residuals	15	25.438	1.696			15	22.146	1.476		

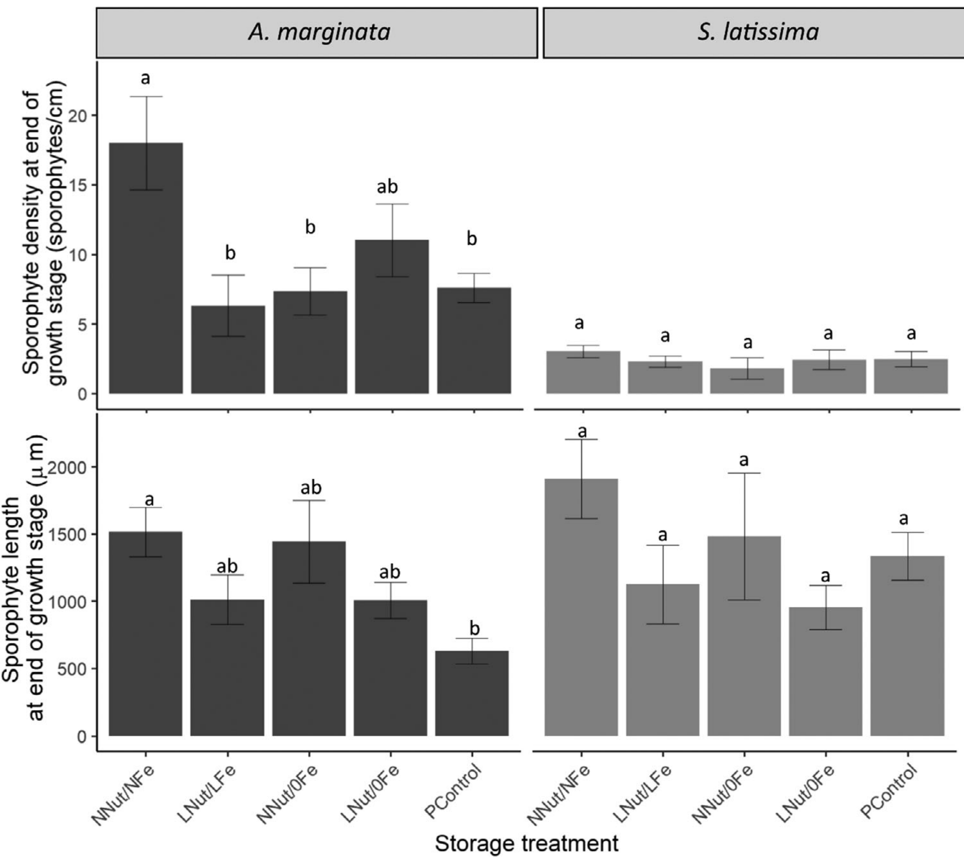

FIGURE 4 Sporophyte density (top) and length (bottom) of *Saccharina latissima* and *Alaria marginata* (Experiment 2.3) in seed storage experiment at the end of the growth stage (Figure 1). Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error. There were four replicates in each combination of storage treatment and species

delayed, therefore reducing dependency on wild sorus and adding the ability to stagger outplantings from the same parent sporophytes. While these results have clear applications for seaweed aquaculture, they also shed light on life history strategies of wild kelp populations and environmental thresholds that may limit or promote growth and reproduction.

To utilize this method, a hatchery would follow a protocol identical to that described in Redmond et al. (2014) except 48 hr after the initial seeding at 12°C, pipes of seeded twine are moved to 4°C for up to 36 additional days of storage or "cold banking." During the time at 4°C, seeded twine should be attended to as specified by Redmond et al. (2014). When preparing to outplant, we suggest moving the "cold banking" seed back into a 12°C environment for 14 to 20 days prior to the desired outplanting date to allow sporophytes to grow to optimal outplanting size.

Our results leading to the "cold banking" procedure highlight the natural overwintering function of kelp gametophytes in the wild. A critical question of kelp ecology is whether gametophytes produced from meiospores in the fall spend the winter waiting for the right conditions for egg production or if gametophytes produce fall juvenile sporophytes, which then overwinter microscopically, ready to grow when ideal conditions returns (Edwards, 2000; Egan et al., 1989; Maxell & Miller, 1996). Our results from Experiment 2.2 suggest it may be a combination of these two strategies for *S. latissima*. As the temperature decreased from 12°C, egg production decreased suggesting that in winter months, gametophytes may reduce their energy investment toward producing eggs. However, levels of

FIGURE 5 Sporophyte density (top) and length (bottom) of *Saccharina latissima* and *Alaria marginata* in seed storage experiment (2.3) at the end of the ocean tank stage. Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error. There were four replicates in each combination of storage treatment and species

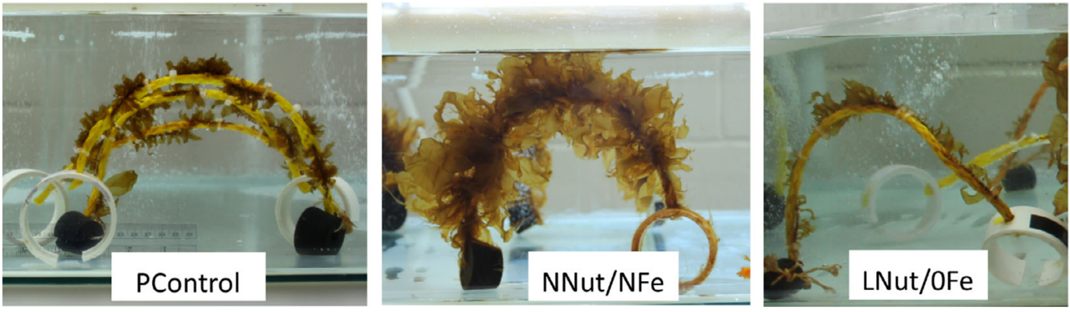

FIGURE 6 Outplanted *Alaria marginata* seeded twine from different treatments near the end of the ocean tank phase (Experiment 2.3). PControl, 20 days after outplanting (taken 8/1/19). NNut/NFe, 22 days after outplanting (taken 8/20/19). Treatment LNut/0Fe, with low sporophyte densities 22 days after outplanting (taken 9/13/19). See Table 1 for treatment conditions

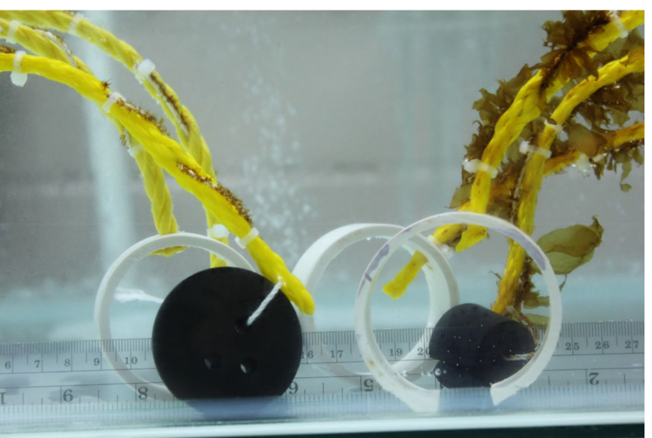

FIGURE 7 Photo demonstrating the ability to stagger outplantings of *Alaria marginata* from the same parent sporophyte with use of the "cold banking" method (Experiment 2.3). A replicate of the NNut/NFe treatment (left) 3 days after outplanting and a PControl replicate (right) 20 days after outplanting in the ocean tank stage (photo taken 8/1/19). Photo illustrates healthy and dense sporophytes in both the control treatment (right) and the treatment that underwent the "cold banking" method in cold temperatures (left). See Table 1 for treatment conditions

sporophyte production at 8°C and 12°C were similar suggesting that during fall months, juvenile sporophytes are still produced at levels similar to summer months, assuming the light under the canopy is within our experimental levels. While not directly measured for this study, seawater near Juneau, at 2 m depth from December 1, 2016 to November 30, 2017 had a mean temperature of 4.54°C (±0.56 *SD*) in winter, 5.90°C (±2.40 *SD*) in spring, 12.17°C (±1.54 *SD*) in summer and 8.98°C (±1.47 *SD*) in fall (unpublished data). The three temperatures we used in Experiment 2.2 represent probable average temperatures for summer (12°C), fall (8°C) and winter (4°C) in this region. Although the present study was not able to address the complex variable effecting microscopic phenology (Bartsch et al., 2008), our results suggest that the overwintering strategy for microscopic *S. latissima* is to diversify the life stages (gametophyte and juvenile sporophytes) contributing to recruitment in the spring. These results are consistent with field studies that found egg production and juvenile sporophyte production of *S. latissima* happened year round (Hsiao & Druehl, 1973). This is inconsistent with other species of brown algae, which have been documented to overwinter strictly in the gametophyte phase and may highlight varying competitive strategies for recruitment (Dayton, 1973). Increased ocean temperatures may accelerate winter gametophytes phenology and could lead to changes in kelp population dynamics (Ladah & Zertuche-González, 2007), altering the availability of critical habitat to ecologically and commercially important organisms (Figure 7).

In Experiment 2.2, we found meiospore release month affected all response measures of *S. latissima* gametophyte growth and reproduction. The significant differences in gametophyte length, sporophyte length and egg production between meiospore release months at 12°C suggest the effect of season on microscopic phenology may be greatest during summertime temperatures and highlighted by the significant interaction of meiospore release month with temperature on sporophyte length and fertility (FFGE). Gametophytes from meiospores released in July grew significantly larger and had more eggs than August gametophytes, but produced significantly smaller sporophytes than those from August. We suggest two likely explanations for the effects of meiospore release month: (a) seasonal variation in phenology and (b) individual sporophyte parent or reproductive fitness (Lewis et al., 2013; Mohring et al., 2013; Muñoz, Hernández-González, Buschmann, Graham, & Vásquez, 2004). Although we do see a difference in variation in all four response variables by season, we do not have any evidence that season may negatively affect seed quality.

In contrast to season and temperature, Experiment 2.3 illustrated the degree to which kelp gametophytes may recover from undesirable conditions. Our results show that the NNut/NFe treatment had the highest densities and sporophyte lengths at the beginning and end of the ocean tank stage for both species and had the shortest SGOT. In the LNut/LFe treatment, *A. marginata* sporophyte density was negatively affected, and both species had longer SGOTs and experienced gametophyte overgrowth compared to NNut/NFe after being moved back to ideal conditions for growth. This result suggests that *A. marginata* gametophytes may be better adapted to recover from low temperatures as opposed to nutrient limitation. This contrasts with studies on the perennial kelp *M. pyrifera* where storage in low nutrients actually had a positive effect on sporophyte growth during recovery in conditions promoting growth when paired with low light (Kinlan, Graham, Sala, & Dayton, 2003; Ladah & Zertuche-González, 2007). In the treatment of NNut/0Fe, *A. marginata* sporophyte density was negatively affected, and both species had longer SGOTs and experienced gametophyte overgrowth after being moved back to ideal conditions for growth. *M. pyrifera* has been described as having similar patterns where egg production from meiospores was hampered by extended storage time in iron deficient media (Lewis et al., 2013). Although iron limitation has clear application in gametophyte clone storage (Lewis et al., 2013; Motomura & Sakai, 1981; Stekoll et al., 2021; Suzuki et al., 1994), using iron limitation for storage of seed produced from wild zoospores has negative effects on seed quality in terms of sporophyte density and gametophyte overgrowth.

Relating our results directly to aquaculture applications, we found that reducing culturing temperature significantly slows gametophyte growth and appears to have little effect on the viability of subsequently outplanted seed. This "cold banking" methodology provides a tool for kelp farmers to delay or stagger outplanting simply by changing the culture temperature of seed. The benefits of delayed or staggered outplantings include the ability to experiment with optimal outplanting times and a staggered harvest from the same parent sporophyte allowing flexibility to preserve food quality of harvested crops and an overall benefit to the kelp mariculture industry. These results also provide insight to the overwintering strategy of kelp gametophytes, suggesting that settled gametophytes may persists during winter months until favorable conditions return in spring. The tight link between gametophyte growth and temperature may also have implications in a warming climate, where warmer winters may mean earlier gametophyte reproduction and sporophyte growth.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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APPENDIX A.

Preliminary incubator trial

Methods

To address the confounding variables of temperature and incubator in Experiment 2.2, we first measured effect of incubator on microscopic kelp growth during a 14-day trial. All incubators and cold room lights were programed for 8:00 a.m. to 4:00 p.m. AK time at 12°C. Gametophyte length was measured under the same protocol outlined in Experiment 2.1. A two-way ANOVA was used to evaluate the effect of irradiance and incubator on gametophyte length with the function aov in R (v3.5.1) (R Core Team, 2018).

Results

Irradiance ($F_{1,80}$, $p = 0.093$) and incubator ($F_{4,80}$, $p = 0.274$) did not affect gametophyte length (Figure A1; Table A1).

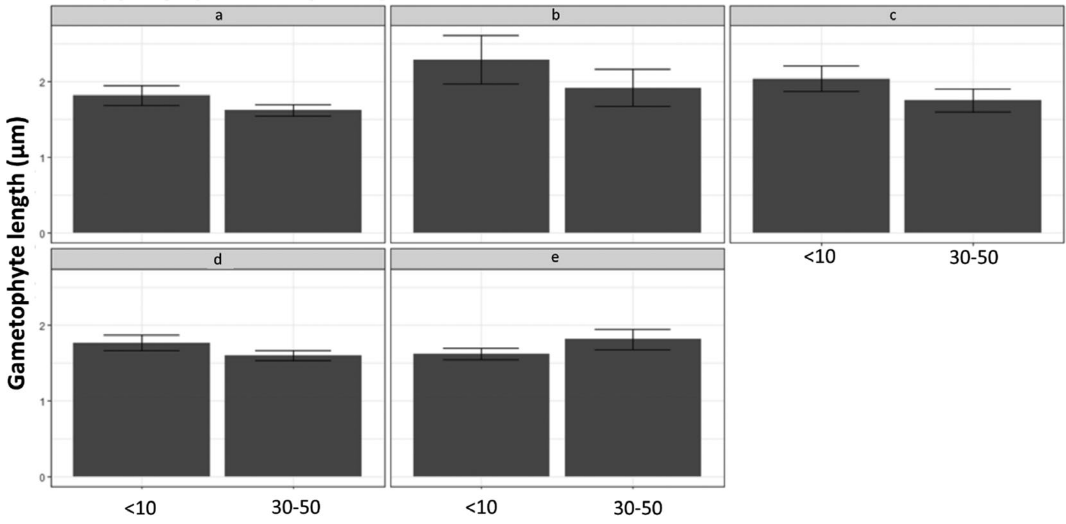

FIGURE A1 Control experiment to evaluate incubator effect on microscopic kelp growth (Experiment 2.1). Gametophytes grown in each incubator (a–e) at 12°C for 14 days under two different irradiance levels (<10 and 30–50 μmol m^{-2} s^{-1}). Error bars represent ±1 standard error

TABLE A1 ANOVA evaluating the effect of irradiance and incubator on gametophyte length during control experiment (Experiment 2.1)

	df	SS	MS	*F* value	*p*
Irradiance	1	0.347	0.347	2.885	0.093
Incubator	4	0.630	0.157	1.310	0.274
Irradiance: incubator	4	0.466	0.116	0.969	0.429
Residuals	80	9.620	0.120		

APPENDIX B.

Incubator effect

Methods

Results from Experiment 2.2.2 show a significant incubator effect for the variables of sporophyte length and FFGE. To investigate the effect of incubator on these variables, we conducted further analysis on paired incubators of the same temperature and meiospore release month to understand more about the source of this effect. Incubators pairs for each meiospores release month set at the same temperature were examined with a two-way ANOVA to evaluate

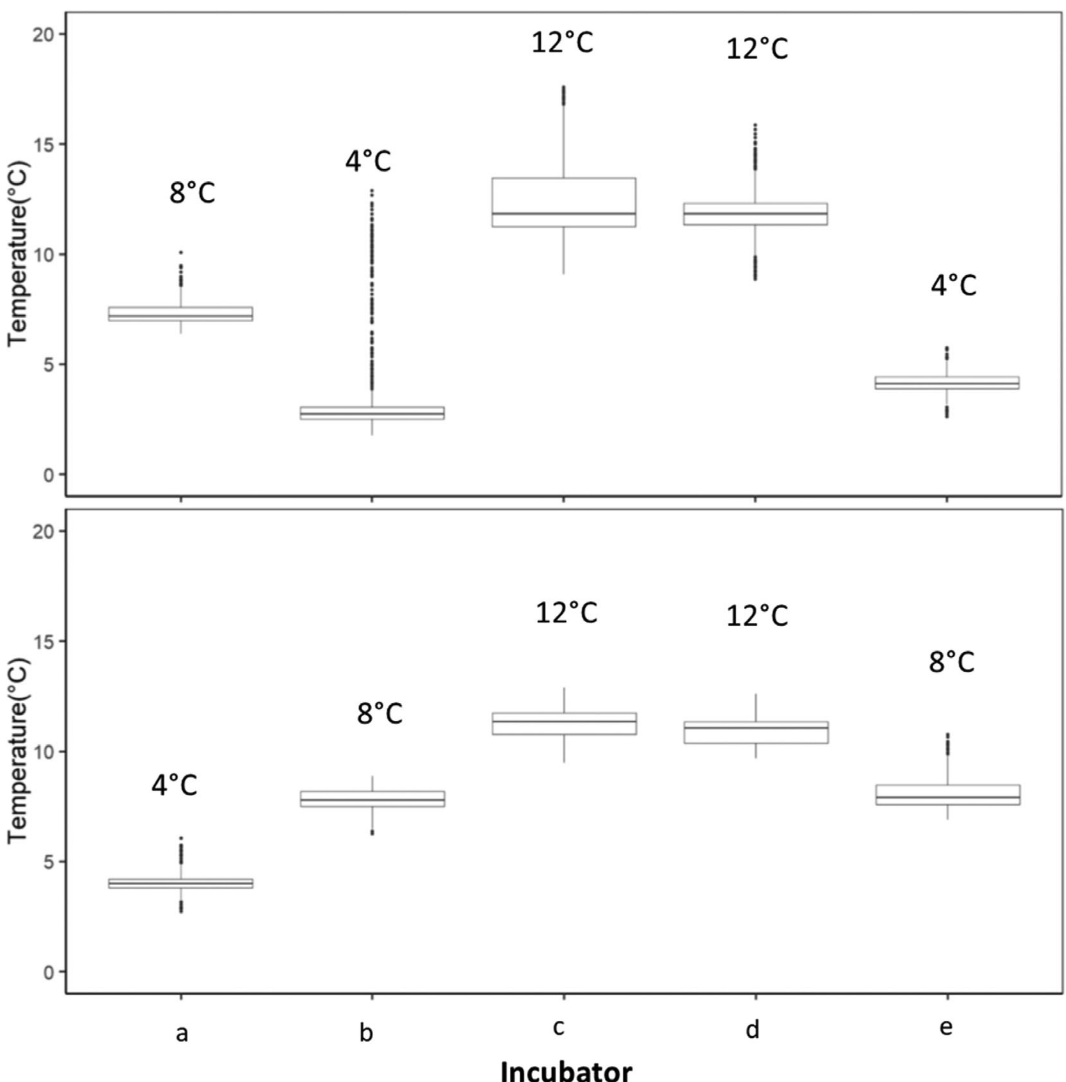

FIGURE B1 FFGE (fraction of female gametophytes with eggs) in each incubator (a–e) and two different irradiance levels (<10 and 30–50 μmol m^{-2} s^{-1}), in July and August meiospores release months (Experiment 2.1). Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error

the effect of irradiance and incubator on sporophyte length and FFGE with the function aov in R (v3.5.1) (R Core Team, 2018).

Results

The effect of incubator on sporophyte length was detectable in the August meiospores release month at 8°C between incubators b and e ($F_{1,29}$, $p = 0.007$), although no significant differences were confirmed by post hoc tests ($p > 0.05$) (Figure B1; Table B1). A detectable incubator effect on FFGE at 4°C in July ($F_{1,31}$, $p = 0.021$) and at 8°C in

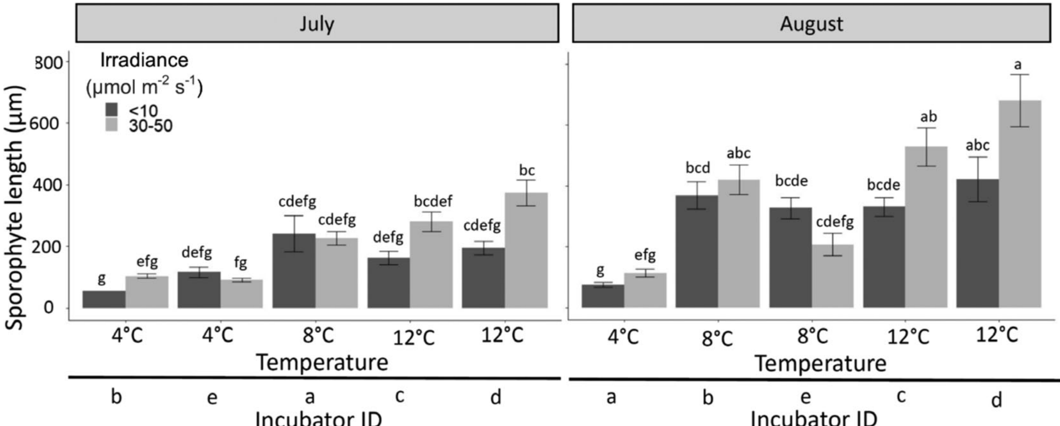

FIGURE B2 Average sporophyte length n each incubator (a–e) and two different irradiance levels (<10 and 30–50 μmol m^{-2} s^{-1}), in July and August meiospores release months (Experiment 2.1). Letters denote statistically similar groups determined by Tukey HSD tests. Error bars represent ±1 standard error

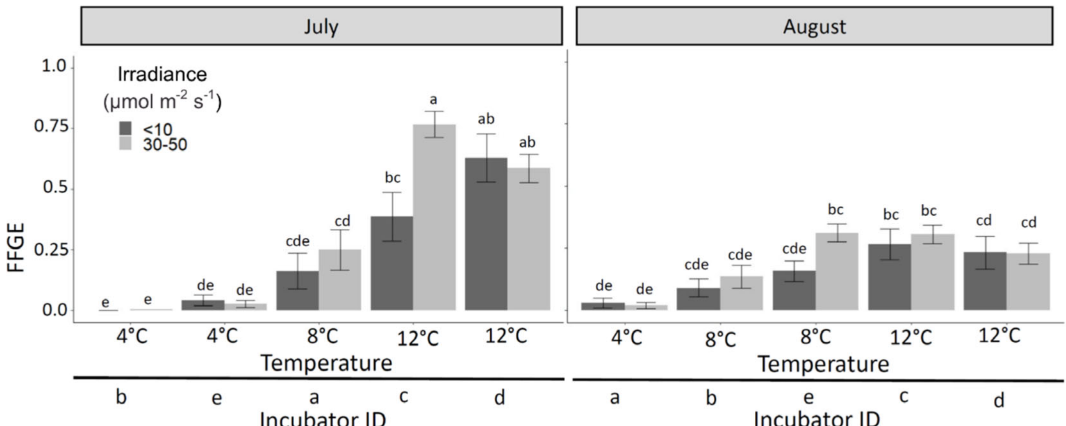

FIGURE B3 Box plot of HOBO logger temperature data in incubators during July (top) and August (bottom) meiospores release months (Experiment 2.1). Temperature values listed above each box plot are the set points for each incubator. Points represent outliers. Box represents the data between the 25th and 75th percentile with middle line as medium

TABLE B1 Comparisons of paired incubators of the same temperatures for the response of sporophyte length (Experiment 2.1)

	df	SS	MS	*F* value	*p*
July meiospore release month					
12°C (incubators c and d)					
Irradiance	1	7.432	7.432	17.427	<0.001
Incubator	1	1.131	1.131	2.653	0.115
Irradiance: incubator	1	0.074	0.074	0.173	0.681
Residuals	28	11.941	0.426		
4°C (incubators b and e)					
Irradiance	1	0.002	0.002	0.012	0.916
Incubator	1	0.049	0.049	0.350	0.570
Irradiance: incubator	1	0.459	0.459	3.285	0.107
Residuals	8	1.118	0.140		
August meiospore release month					
12°C (incubators c and d)					
Irradiance	1	6.856	6.856	10.253	0.003
Incubator	1	1.164	1.164	1.741	0.198
Irradiance: incubator	1	0.101	0.101	0.151	0.701
Residuals	28	18.724	0.669		
8°C (incubators b and e)					
Irradiance	1	0.862	0.862	1.624	0.213
Incubator	1	4.526	4.526	8.528	0.007
Irradiance: incubator	1	2.360	2.360	4.447	0.044
Residuals	29	15.390	0.531		

August was documented ($F_{1,31}$, $p = 0.005$) yet the effects of incubator were not confirmed through post hoc comparisons ($p > 0.05$) (Figure B2; Table B2). These differences may be due to incubators set at the same temperature, acting differently ranges among replicate incubators set at the same temperature (Figure B3).

TABLE B2 Comparisons of paired incubators of the same temperatures for the response of Fraction of female gametophytes with eggs (FFGE) (Experiment 2.1)

Factors of interest	*df*	SS	MS	*F* value	*p*
July meiospore release month					
12°C (incubators c and d)					
Irradiance	1	0.481	0.481	4.813	0.036
Incubator	1	0.002	0.002	0.016	0.899
Irradiance: incubator	1	0.567	0.567	5.676	0.024
Residuals	31	3.097	0.100		
4°C (incubators b and e)					
Irradiance	1	0.001	0.001	0.049	0.825
Incubator	1	0.087	0.087	5.888	0.021
Irradiance: incubator	1	0.008	0.008	0.544	0.466
Residuals	31	0.458	0.015		
August meiospore release month					
12°C (incubators c and d)					
Irradiance	1	0.007	0.007	0.158	0.694
Incubator	1	0.069	0.069	1.515	0.227
Irradiance: incubator	1	0.004	0.004	0.080	0.779
Residuals	32	1.453	0.045		
8°C (incubators b and e)					
Irradiance	1	0.154	0.154	3.809	0.060
Incubator	1	0.380	0.380	9.377	0.005
Irradiance: incubator	1	0.053	0.053	1.317	0.260
Residuals	31	1.257	0.041		