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RESEARCH ARTICLE

Effects of temperature and nutrients on microscopic stages of the bull kelp (*Nereocystis luetkeana*, Phaeophyceae)

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Abstract

Warming ocean temperatures have been linked to kelp forest declines worldwide, and elevated temperatures can act synergistically with other local stressors to exacerbate kelp loss. The bull kelp *Nereocystis luetkeana* is the primary canopy-forming kelp species in the Salish Sea, where it is declining in areas with elevated summer water temperatures and low nutrient concentrations. To determine the interactive effects of these two stressors on microscopic stages of *N. luetkeana*, we cultured gametophytes and microscopic sporophytes from seven different Salish Sea populations across seven different temperatures (10–22°C) and two nitrogen concentrations. The thermal tolerance of microscopic gametophytes and sporophytes was similar across populations, and high temperatures were more stressful than low nitrogen levels. Additional nitrogen did not improve gametophyte or sporophyte survival at high temperatures. Gametophyte densities were highest between 10 and 16°C and declined sharply at 18°C, and temperatures of 20 and 22°C were lethal. The window for successful sporophyte production was narrower, peaking at 10–14°C. Across all populations, the warmest temperature at which sporophytes were produced was 16 or 18°C, but sporophyte densities were 78% lower at 16°C and 95% lower at 18°C compared to cooler temperatures. In the field, bottom temperatures revealed that the thermal limits of gametophyte growth (18°C) and sporophyte production (16–18°C) were reached during the summer at multiple sites. Prolonged exposure of bull kelp gametophytes to temperatures of 16°C and above could limit reproduction, and therefore recruitment, of adult kelp sporophytes.

KEYWORDS
climate change, gametophyte, kelp, microscopic sporophyte, nitrate, nutrients, temperature

INTRODUCTION

Over the past century, the global mean sea surface temperature has increased by 0.88°C and the frequency of marine heatwaves has doubled, exposing key species in coastal marine ecosystems to temperatures beyond their tolerance limits (Cooley et al., 2022). Kelp, large brown algae in the order Laminariales, form vast underwater forests in temperate and subpolar regions, occupying up to 28% of the world's coastlines (Starko et al., 2021). Kelps are foundation species, supporting biodiversity hotspots through habitat provisioning

Abbreviations: N, nitrogen; NH_4, ammonium; NO_3, nitrate; P, phosphorous; PO_4, phosphate.

and tremendously high primary productivity (Duarte et al., 2022). More than one third of the world's kelp forests have declined in abundance over the past 50 years (Krumhansl et al., 2016), with further declines reported recently from many regions (Filbee-Dexter & Wernberg, 2018; Smale, 2020). These declines have been linked to ocean warming and marine heatwaves around the world (Arafeh-Dalmau et al., 2019; Berry et al., 2021; Filbee-Dexter et al., 2020; Filbee-Dexter & Wernberg, 2018; Kumagai et al., 2018; Rogers-Bennett & Catton, 2019; Starko et al., 2022). Sea surface temperatures will continue to increase, and marine heatwaves will become even more frequent, so we must be prepared with a comprehensive understanding of the consequences of ocean warming for kelp forest ecosystems.

Many marine organisms have complex life cycles with distinct free-living stages that may be exposed to and respond differently to changing environmental conditions, so it is critical to determine the thermal vulnerability of all life cycle stages. Kelps have a biphasic life cycle with two free-living stages: microscopic, haploid gametophytes and macroscopic, diploid sporophytes. Thermal tolerances can differ across life history stages of kelp (Harley et al., 2012), yet most studies have tested the effects of environmental stressors only on adult sporophytes, leaving a crucial knowledge gap in our understanding of how stressors impact several early life stages—zoospores, gametophytes, and juvenile sporophytes (Hollarsmith et al., 2022). A recent review suggests that kelp gametophytes can tolerate a wider range of temperatures compared to sporophytes (Veenhof et al., 2022), including higher temperatures than those usually experienced in the environment (tom Dieck, 1993). Kelp gametophytes may persist on the benthos even when conditions are unfavorable for sporophytes, playing a critical role in population resilience by serving as microscopic seed banks (Carney, 2011; Edwards, 2022).

Due to upwelling and stratification, high seawater temperatures are often correlated with low nitrogen levels in coastal ecosystems (Palacios et al., 2013). Climate change is altering ocean circulation and stratification patterns, with continued increases in stratification leading to declines in upper-ocean nutrients (Cooley et al., 2022). High temperatures and low nitrogen concentrations are both physiologically stressful for kelp, and experiments with adult sporophytes have shown that these two stressors can act synergistically with nitrogen limitation worsening the impact of high temperatures (Fernández et al., 2020; Gerard, 1997; Schmid et al., 2020; Umanzor et al., 2021). However, only a few studies have examined the interactive effects of temperature and nitrogen on kelp gametophytes or juvenile sporophytes. Across 12 kelp species, gametophyte survival and sporophyte production were more severely limited by high temperatures (18°C) than by low nitrogen levels (1 μM nitrate; Muth et al., 2019). Similarly, gametophyte cultures of *Laminaria digitata* and *Ecklonia radiata* did not produce sporophytes at temperatures above 18°C and 22°C, respectively, regardless of nitrogen levels (Mabin et al., 2013; Martins et al., 2017). In contrast, other studies observed that embryonic sporophyte production was limited by the availability of nitrogen across temperatures (Matson & Edwards, 2007; Shukla & Edwards, 2017).

The bull kelp, *Nereocystis luetkeana*, grows from central California to the Aleutian Islands in the northeast Pacific Ocean. *Nereocystis luetkeana* is the primary canopy-forming kelp species in the Salish Sea, a fjordal estuary that encompasses Puget Sound, the Strait of Juan de Fuca, and the Strait of Georgia. The Salish Sea is warming rapidly, with projections of a 1.5–3°C increase in mean water temperature by the end of the 21st century (Amos et al., 2015; Khangaonkar et al., 2019; Riche et al., 2014). Bull kelp declines have been linked to ocean warming in multiple locations. In South Puget Sound, bull kelp forests have declined by more than 60% since the 1870s, and these losses are correlated with elevated summer water temperatures and low nutrient concentrations (Berry et al., 2021). In British Columbia, bull kelp declines have occurred primarily along warmer, wave-sheltered coastlines (Starko et al., 2019, 2022). A marine heatwave in 2013–2014 was followed by a 50% decline in bull kelp canopy cover on the outer coast of Washington, but the bull kelp canopy recovered by the next year (Tolimieri et al., 2023). While kelp recovery occurred on the outer coast, kelp forests are still in decline in the warmer, more inland waters of the Salish Sea.

During the annual life cycle of *Nereocystis luetkeana*, adult sporophytes become reproductive during the late spring and summer (Maxell & Miller, 1996), releasing microscopic zoospores that settle to the benthos and germinate into male and female gametophytes. However, some adult sporophytes that overwinter may continue to reproduce throughout the fall and winter and into the following spring (Ulaski & Konar, 2021). Sexual reproduction occurs when the male gametophyte releases sperm that fertilizes the female oogonium, which develops into a juvenile sporophyte. Gametophytes of *N. luetkeana* can germinate and survive between 5 and 21°C, but germination and growth rates have been shown to decline above 17°C (Lind & Konar, 2017; Muth et al., 2019; Schiltroth, 2021; Vadas, 1972). The production of juvenile sporophytes through sexual reproduction is limited to temperatures below 20°C (Vadas, 1972), but information on the thermal window for juvenile sporophyte production by *N. luetkeana* is lacking, as most studies have only tested a few temperatures.

Finally, both thermal history and genetic differentiation can cause population-specific responses to ocean warming (Liesner et al., 2020; Sánchez de Pedro

et al., 2022). Kelp responses to warming can depend on previous thermal exposure (Gauci et al., 2022), which tends to differ across populations, leading to intraspecific differences in thermal tolerance (Becheler et al., 2022; Martins et al., 2020; Schimpf et al., 2022; Strasser et al., 2022). The Salish Sea is a large and complex estuarine system, with sea surface temperatures that can span >8°C across sites that are <100 km apart, depending on the tides, currents, river inputs, and distance from the Pacific Ocean (MacCready et al., 2021). Therefore, it is critical to conduct thermal tolerance studies across multiple populations, as they may respond differently to elevated temperatures.

To determine the population-specific temperature thresholds for survival, growth, and reproduction of early life stages of *Nereocystis luetkeana* within the Salish Sea, we cultured gametophytes from seven different sites across seven different temperatures, spanning 10–22°C, and at two different nutrient concentrations (high v. low). In addition, we tracked subtidal temperatures in the kelp forest at each site to compare the critical temperatures for gametophyte growth and sporophyte production with the in situ temperatures that each population experienced. As nitrogen can buffer the effect of high temperatures for adult kelp sporophytes (Fernández et al., 2020; Gerard, 1997), we hypothesized that high nitrogen concentrations would allow microscopic stages to survive at higher temperatures. In addition, we hypothesized that populations from warmer water sites would have a higher resilience to elevated temperatures.

MATERIALS AND METHODS

Field sampling and temperature monitoring across populations

Mature sori (reproductive patches) were collected from eight different bull kelp (*Nereocystis luetkeana*) populations within the Salish Sea in Washington State, United States. Populations were selected to span a large geographic area that corresponds to differences in seawater temperatures, from South Puget Sound (Squaxin Island, Tacoma Narrows) to Central Puget Sound (Vashon Island, Lincoln Park, Edmonds) and North Puget Sound/the San Juan Islands (Turn Rock, Gordon Island, Cherry Point; Figure 1). Site names, GPS coordinates, and sori collection dates are listed in Table 1. We conducted two experiments to accommodate all eight

FIGURE 1 Map of the eight study sites in the Salish Sea, Washington, USA.

TABLE 1 Location of study sites, including GPS coordinates, dates sampled, and mean (±SE) surface seawater nutrient concentrations at each site.

Location	GPS (latitude, longitude; °)	Date	NO_3 (μM)	NO_2 (μM)	NH_4 (μM)	PO_4 (μM)
Squaxin Island	47.168, −122.896	6/29/22	8.66 ± 0.26	0.66 ± 0.02	2.56 ± 0.05	1.62 ± 0.01
Tacoma Narrows	47.296, −122.532	6/29/22	15.43 ± 0.15	0.78 ± 0.01	1.22 ± 0.03	1.77 ± 0.01
Vashon Island	47.478, −122.447	6/28/22	3.24 ± 0.28	0.30 ± 0.02	0.58 ± 0.05	1.11 ± 0.03
Lincoln Park	47.535, −122.398	6/28/22	2.58 ± 0.17	0.27 ± 0.01	0.90 ± 0.03	1.06 ± 0.03
Edmonds	47.817, −122.379	8/8/22	2.07 ± 0.11	0.26 ± 0.01	0.28 ± 0.01	0.80 ± 0.01
Turn Rock	48.535, −122.965	8/9/22	15.46 ± 0.82	0.36 ± 0.01	0.9 ± 0.10	1.43 + 0.07
Gordon Island	48.730, −123.021	8/9/22	9.78 ± 0.07	0.2 ± 0.00	0.53 ± 0.03	1.01 ± 0.01
Cherry Point	48.855, −122.731	8/8/22	0.64 ± 0.15	0.06 ± 0.01	0.18 ± 0.03	0.5 ± 0.02

Note: Nutrients were sampled on the same day that reproductive sori were collected for gametophyte culturing.

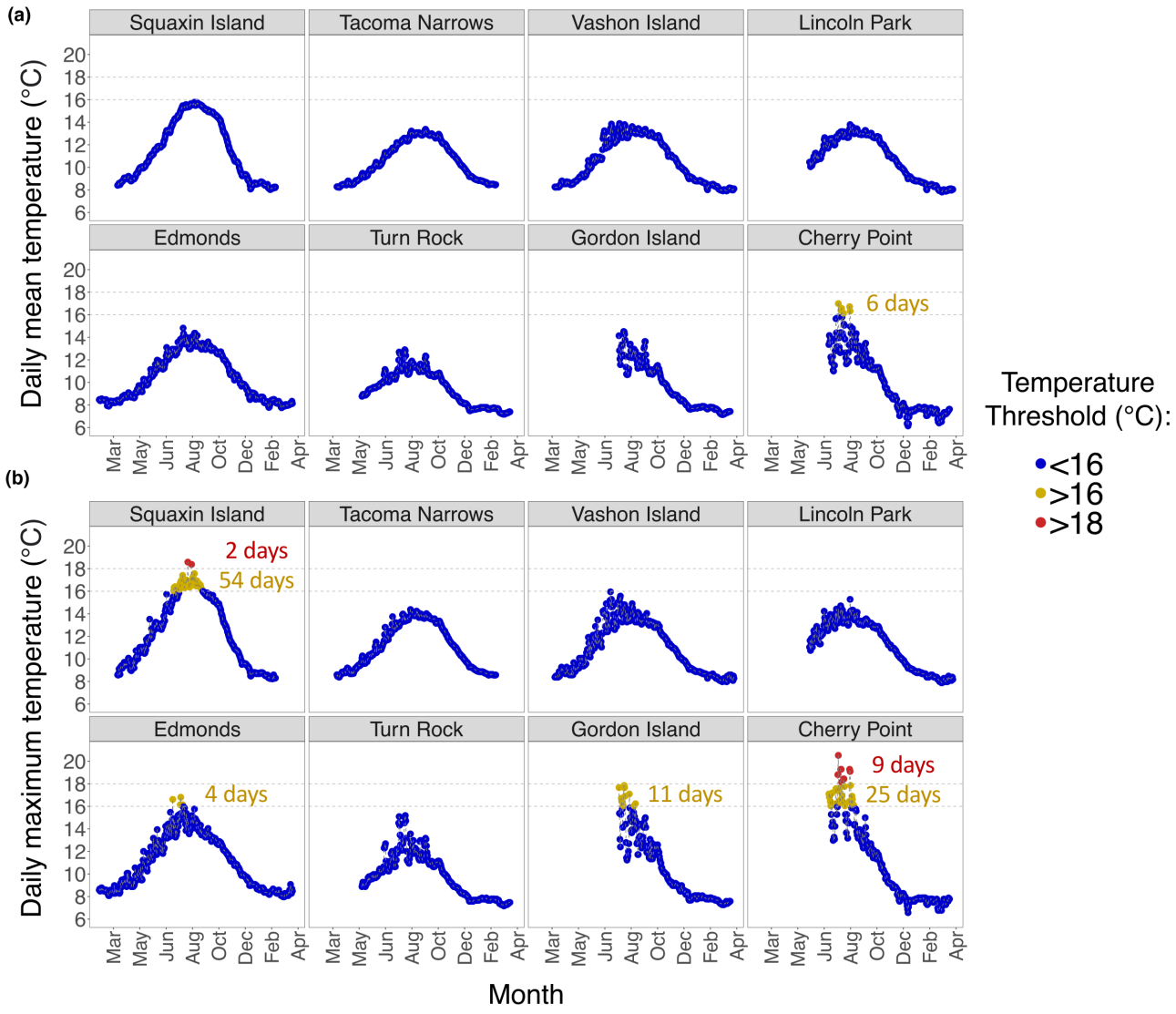

FIGURE 2 (a) Daily mean temperatures from the study sites, measured at the bottom (−3 to −5 m relative to MLLW) adjacent to kelp forests. (b) Daily maximum temperatures from the same sites and depths. Temperatures are shaded according to thresholds of <16, >16, and >18°C. For sites where temperature thresholds were exceeded, the number of total days above 16 or 18°C is indicated on the graph.

populations: the first set included sori from Squaxin Island, Tacoma Narrows, Vashon Island, and Lincoln Park collected June 28–29, 2022, and the second set included sori from Edmonds, Turn Rock, Gordon Island, and Cherry Point collected August 8–9, 2022. Sori from Cherry Point did not release enough zoospores

to be included in the experiment, likely because we had just missed a period of natural zoospore release at that site. However, we still included temperature data from Cherry Point. At each site, sori from 15 different individuals were collected and kept cool with ice packs and refrigeration during transportation to Friday Harbor Labs. Triplicate surface seawater samples were collected from within the kelp forest at each site, filtered through 0.7-μm glass-fiber filters, and frozen until analysis. Seawater inorganic nutrient concentrations (NO_3^-, NO_2^-, NH_4^+, PO_4^-) were quantified at the University of Washington Marine Chemistry Lab using standard methods (UNESCO, 1994). Subtidal temperature and pressure sensors (HOBO Onset Water Level Logger, U20L-02) were installed near the seafloor at each site via SCUBA between −3 and −5 m depth relative to mean lower low water (MLLW). Sensors were swapped every 3–5 months for 1 year.

Kelp gametophyte culturing across temperatures and nitrogen levels

Each individual sorus was cut to 3 × 3 cm, gently wiped with a paper towel, sterilized for 30 s with Betadine iodine solution, rinsed with 1.0 μm filtered and autoclaved seawater, and placed between layers of seawater-dampened paper towels for 2 hrs in the refrigerator. Sori were then placed into beakers of sterilized seawater overnight at 10°C to induce zoospore release. Within each population, 15 ripe sori from different individuals were combined during zoospore release to ensure genetic diversity. Zoospore concentrations were quantified with a hemocytometer. To ensure optimal densities for juvenile sporophyte production, we first conducted a density experiment by plating zoospores from one population (Turn Rock) across six different densities ranging from 60 to >2,100 spores · mL^{-1}. Our assay demonstrated that a density of 530 spores · mL^{-1} ultimately yielded the greatest number of juvenile sporophytes (Figure S1 in the Supporting Information). Therefore, for each population, hemocytometer counts were used to standardize the zoospore densities in our experiments to 530 spores · mL^{-1}. Gametophytes were cultured in 12-well plates with 5 mL media in each well, under both high and low nutrient levels, across seven temperatures (six replicate wells per treatment).

Nutrient media was prepared to achieve realistic N and P concentrations from this region, as standard F/2 growth media has 880 μM $NaNO_3$ and 36 μM NaH_2PO_4, roughly 40–80 and 18–36 times the natural amount of NO_3 and PO_4 in coastal marine seawater, respectively. Our low N nutrient treatment was prepared with 1.0 μm filtered and autoclaved seawater enriched with F/2 concentrations of trace metals and B vitamins but no additional N or P. The high N nutrient treatment included the above, plus 30 μM $NaNO_3$, 1 μM NH_4Cl, and 2 μM NaH_2PO_4. Media was changed weekly for 5 weeks. Based on analyzed samples of nutrient media, the high N media started with approximately 50 μM NO_3, 1.0 μM NH_4, and 4.0 μM PO_4, while low N media started with approximately 20 μM NO_3, 0.1 μM NH_4, and 2.0 μM PO_4. However, after 1 week of gametophyte growth across temperatures (between days 10 and 17), high N spent media was still nitrogen-replete with a mean (±SD) of 7.21 ± 7.48 μM NO_3, 0.14 ± 0.04 μM NH_4, and 0.56 ± 0.37 μM PO_4, while low N spent media contained only 0.59 ± 0.68 μM NO_3, 0.10 ± 0.03 μM NH_4, and 0.12 ± 0.06 μM PO_4.

Experimental temperatures of 10, 12, 14, 16, 18, 20 and 22°C were chosen to span a range of environmental temperatures experienced in this region (Figure 2) or that are projected to occur in the next century (Khangaonkar et al., 2019). Temperatures were maintained inside a cold room, where sealed gametophyte culture plates were placed into seven different 5-gallon aquaria equipped with titanium heaters, temperature controllers, and micropumps to maintain evenly heated water. Plates containing gametophyte cultures were placed into the aquaria so that the bottom halves of the plates were in contact with the heated water, but the plates were not submerged. Plates were parafilm-sealed to prevent evaporation and changes in salinity. Temperatures were recorded inside of the sealed culture plate wells at random intervals with Pyroscience self-adhesive temperature sensor spots (#TPSP5-ADH). Mean (±SD) treatment temperatures were 10.4 ± 0.5, 11.6 ± 0.5, 14.1 ± 0.4, 15.9 ± 0.5, 17.7 ± 0.5, 20.3 ± 0.5, and 21.7 ± 0.3°C. Zoospores were plated directly into each treatment, and temperatures were held constant from zoospore germination and gametophyte growth through sporophyte production. Gametophyte cultures were kept for 6 weeks under an irradiance level of 40–60 photosynthetically active radiation (PAR; μmol · m^{-2} · s^{-1}) with cool white (5000 k) LED lights and a 15:9 h light:dark cycle, as Washington receives an average of about 15 h of daylight during the summer months.

Gametophyte and juvenile sporophyte response metrics

To quantify size and growth rates, we took photographs of the gametophytes and juvenile sporophytes using a Nikon Eclipse TE2000-U inverted microscope equipped with an AmScope microscope camera (MU1003B). Gametophyte photos were taken on day 10, and weekly after that for 3 weeks (days 17, 24, 31). Juvenile sporophyte photos were taken on days 32 to 34. Photos of three random fields of view were taken from each replicate well, for a total of 18 photos per treatment (6 replicate wells × 3 photos). We used a custom ImageJ macros script with batch processing to convert all images to black and white, then the magic

wand tool in ImageJ to measure the area of individual gametophytes. A calibration slide was used to convert to mm^2 for each magnification. A similar ImageJ macros script was used to convert sporophyte images, but sporophyte areas were determined by hand-drawing polygons around each individual sporophyte using ImageJ. Gametophyte growth rates were quantified as the change in mean gametophyte size per well over time; we analyzed the difference in size between days 10 and 17 to capture growth rates before embryogenesis, which likely impacts growth rates.

After 40 d, the total number of juvenile sporophytes in each replicate well was counted. At the end of the 6 weeks (42 d), gametophytes were counted from three random fields of view per replicate well and scaled up to the total number per well. In the case of extremely low sporophyte densities, all sporophytes in a well were counted, but most were counted with three random fields of view per well and scaled up to the total number.

Statistical analysis

We used linear mixed-effects models in R (version 4.2.2) with the package nlme to test the fixed effects of temperature, nutrients, and source population, as well as their interactions, on each response variable, which included gametophyte growth rates, sporophyte size, gametophyte density, and sporophyte density. All experiments were conducted in 12-well plates, so well was included as a random factor in all mixed-effects models. Following significant model outcomes, pairwise tests were conducted using the package emmeans, while controlling for multiple comparisons with the Tukey method.

RESULTS

Seawater temperatures & nutrient concentrations across sites

Seawater temperatures, measured at the bottom at a standardized depth within or adjacent to kelp forests, remained cool during the winter and spring at all sites and warmed slowly throughout the summer, peaking in August (Figure 2). Daily mean seawater temperatures remained below 16°C at all sites except Cherry Point, which exceeded 16°C on 6 d in July and August, including two consecutive periods from August 2–3 and 20–21 (Figure 2). Daily maximum seawater temperatures exceeded 16°C at Squaxin Island on 54 d, Edmonds on 4 d, Gordon Island on 11 d, and Cherry Point on 25 d (Figure 2). At the two warmest sites, Squaxin Island and Cherry Point, daily maximum seawater temperatures exceeded 18°C on 2 and 9 d, respectively (Figure 2). The highest recorded temperature was 20.5°C at Cherry Point on July 27. Daily maximum temperatures exceeding 16°C occurred consecutively at Squaxin Island for 50 d from July 25 to September 12. During that period, daily mean temperatures at Squaxin Island averaged 15.45 ± 0.22°C. In contrast, warm periods at Cherry Point and Gordon Island were periodic, punctuated by days with cooler water temperatures (Figure 2).

Surface seawater nutrient concentrations differed across sites (Table 1), although samples were limited to a single time point corresponding to sori collection dates. Seawater nitrate (NO_3) concentrations were below 5 μM at sites in Central Puget Sound (Vashon Island, Lincoln Park, Edmonds), while nitrate at Cherry Point was <1 μM. Sites in the San Juan Islands (Turn Rock, Gordon Island) and the Tacoma Narrows site had the highest nitrate concentrations of 10–15 μM (Table 1).

Gametophyte responses to temperature and nitrogen across populations

Gametophytes from all populations displayed strong responses to temperature, with thermal performance curves that show slower growth rates at cooler temperatures (10–12°C), maximum growth rates at warmer temperatures (14–18°C), and a sharp decline to zero growth at 20 and 22°C (Figure 3, Table 2). Only a few gametophytes germinated at 20 and 22°C, mainly from Turn Rock and Gordon Island, and they died by day 17 (Figure S2 in the Supporting Information). After that, gametophytes did not survive at 20 or 22°C from any population, regardless of nitrogen levels (Figure S2, Figure 3). After 6 weeks of growth across temperature and nitrogen treatments, the density of gametophyte cultures also displayed strong temperature dependence (Figure 4a). Gametophyte densities were highest at cooler temperatures from 10 to 16°C, significantly lower at 18°C, and dropped to zero at 20–22°C, regardless of source population or nitrogen treatment (Figure 4a). Across all populations and nitrogen levels, gametophyte densities were, on average, 59% lower at 18°C compared with the mean densities at cooler temperatures (10–14°C). Peak gametophyte densities occurred at 10–12°C for many populations, although these were not always significantly different from densities at 14 or 16°C (Figure 4a). Gametophyte growth rates were highest when gametophyte densities were low (Figure S3 in the Supporting Information), and the low density of gametophytes at 18°C may explain why high gametophyte growth rates occurred at 18°C for some populations.

Overall, gametophytes were much more limited by high temperatures than by low nitrogen concentrations, and temperatures above 18°C were lethal

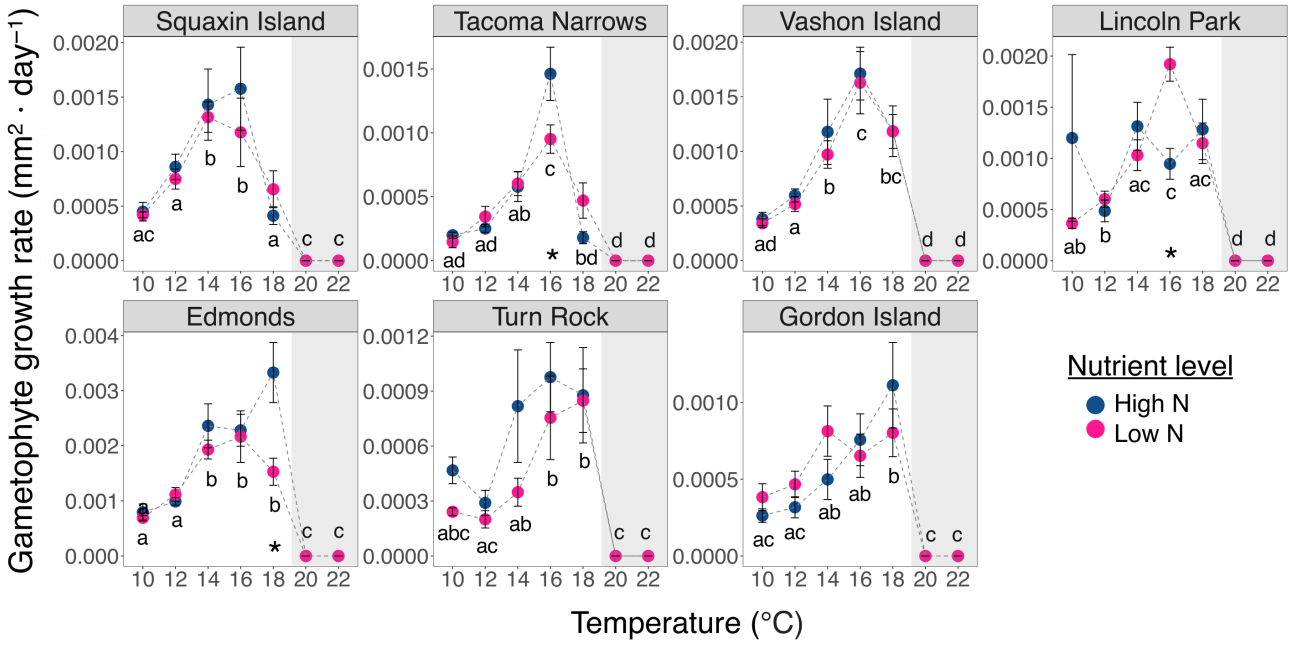

FIGURE 3 Mean (±SE) gametophyte growth rates (mm^2 per day) across temperatures, calculated as the difference in mean size over 1 week (between days 10 and 17). Graphs are separated by source population, with temperature on the *x*-axis and nitrogen treatment indicated by differently shaded points. Note variation in *y*-axes among populations. Within each population, different letters indicate statistically significant post-hoc comparisons ($p < 0.05$) of mean growth rates across temperatures, and asterisks (*) indicate statistically significant post-hoc comparisons ($p < 0.05$) of mean growth rates between nitrogen treatments at a given temperature. The gray panels indicate temperatures at which gametophyte growth rates were zero.

TABLE 2 Results of mixed-effects models testing the effects of temperature, nitrogen, and source population, as well as their interactions, on gametophyte and sporophyte response metrics.

		Gametophyte growth rate		Gametophyte density		Sporophyte density		Sporophyte size	
Factor	***df***	***F* value**	***p* value**	***F* value**	***p* value**	***F* value**	***p* value**	***F* value**	***p* value**
Temperature	6	124.54	<0.001	698.20	<0.001	153.78	<0.001	5.70	<0.001
Population	6	37.06	<0.001	266.37	<0.001	206.61	<0.001	7.54	<0.001
Nitrogen	1	5.71	0.038	1.49	0.25	22.67	<0.001	11.17	0.001
Temp*Population	36	5.69	<0.001	32.37	<0.001	38.27	<0.001	1.10	0.33
Temp*Nitrogen	6	1.20	0.30	1.13	0.35	6.49	<0.001	2.67	0.016
Population*Nitrogen	6	1.52	0.17	0.34	0.92	5.55	<0.001	1.90	0.08
Temp*Population*Nitrogen	36	2.11	<0.001	2.69	<0.001	3.00	<0.001	0.51	0.98

to gametophytes. There were significant interactions between temperature, nitrogen, and population on gametophyte growth rates (mixed-effects model, $p < 0.001$; Table 2). Growth rates responded to nitrogen, but only within a few populations at 16 or 18°C, and the high nitrogen treatment was associated with both higher and lower growth rates (Figure 3). There was no overall effect of nitrogen or interactive effect of temperature and nitrogen on gametophyte density (mixed-effects model, $p > 0.05$; Table 2). While there was a significant interactive effect of all three factors (temperature, nitrogen, and source population) on gametophyte density (Table 2), only four populations responded to nitrogen at certain temperatures (Figure 4a). When the effect of nitrogen was significant, gametophyte densities were higher under high N, except for Edmonds and Turn Rock at 10°C, where lower gametophyte density was displayed at high N (Figure 4a).

There were also significant effects of source population, as well as the interaction between temperature and source population, on both gametophyte growth rate and density (mixed-effects models, $p < 0.001$; Table 2). Even though all cultures were seeded with the same initial density of zoospores, populations displayed differences in the magnitude of gametophyte densities and growth rates, as seen by the varying *y*-axis scale (Figures 3 and 4a). Gametophyte densities were approximately twice as

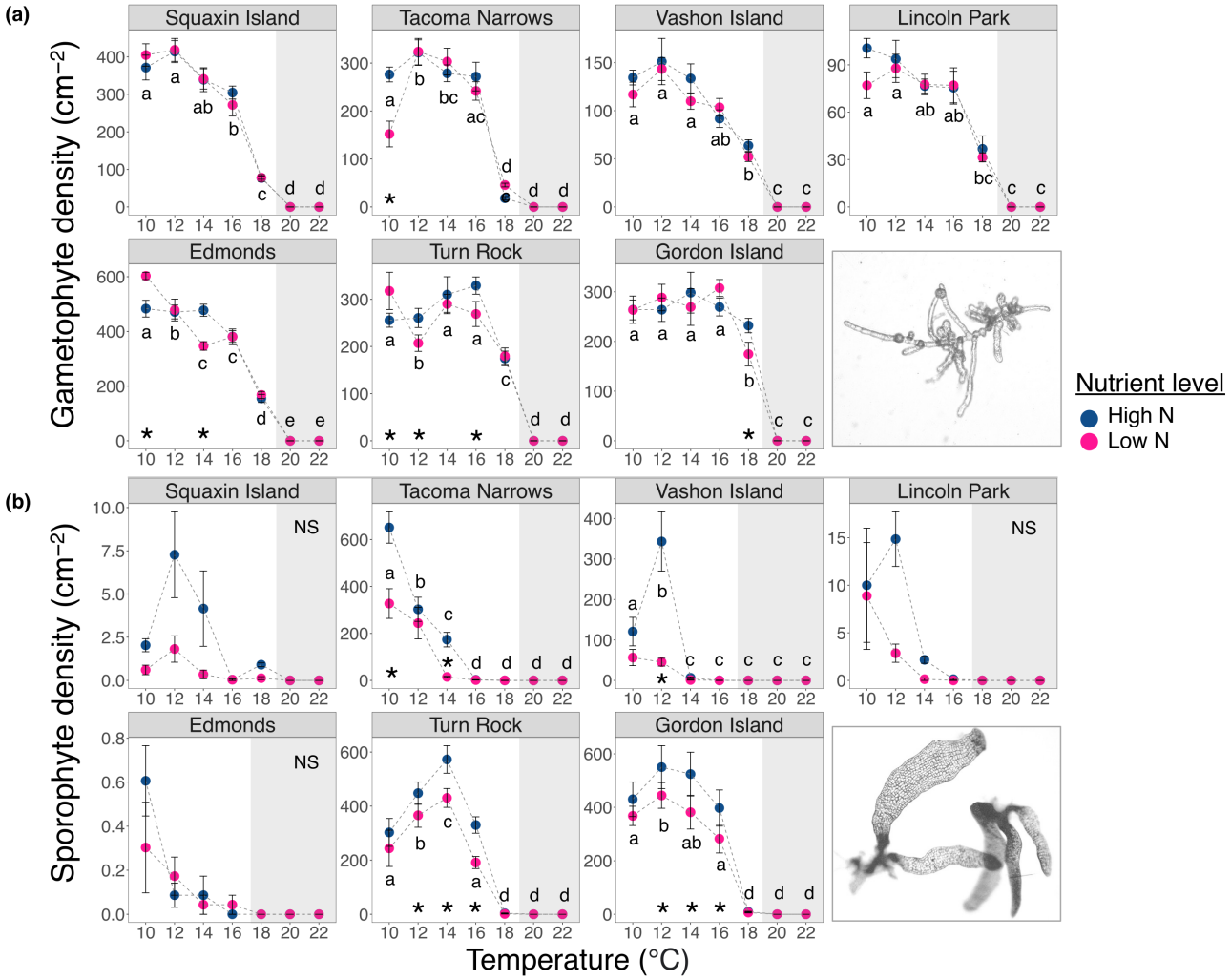

FIGURE 4 (a) Mean (±SE) gametophyte density (gametophytes per cm^2) across temperatures after 42 d of growth. (b) Mean (±SE) sporophyte density (sporophytes per cm^2) across temperatures after 40 d of growth. Graphs are separated by source population, with temperature on the *x*-axis and nitrogen treatment indicated by differently shaded points. Within each population, different letters indicate statistically significant post-hoc comparisons ($p < 0.05$) of mean density across temperatures, and asterisks (*) indicate statistically significant post-hoc comparisons ($p < 0.05$) of mean density between nitrogen treatments at a given temperature. For three populations with low sporophyte densities in (b), post hoc pairwise comparisons of means across temperatures were not significant (NS). The gray panels indicate temperatures at which gametophyte or sporophyte densities were zero.

high in the Edmonds population compared with Tacoma Narrows, Turn Rock, Gordon Island, and Squaxin Island populations, while density was lowest in populations from Vashon Island and Lincoln Park (Figure 4a). Since gametophyte density and growth rate are inversely related (Figure S3a), growth was faster in populations with lower gametophyte densities such as Vashon Island and Lincoln Park and slower in populations with higher densities such as Edmonds, Squaxin Island, Turn Rock, and Gordon Island (Figure 3). Despite variation in gametophyte densities and growth rates among populations, thermal tolerance was similar across populations; gametophyte densities declined at 18°C and above across all populations (Figures 3 and 4a). Finally, we observed abnormally rounded cells on many gametophytes at 18°C; this occurred across populations and nitrogen treatments (Figure S4 in the Supporting Information).

Production of sporophytes in response to temperature and nitrogen across populations

The density of sporophytes produced by gametophyte cultures responded significantly to temperature, nitrogen, source population, and their interactions (mixed-effects model, $p < 0.001$; Table 2). The highest temperature at which any sporophytes were produced was 16°C for three out of seven populations (Vashon Island, Lincoln Park, and Edmonds) and 18°C for four populations (Squaxin Island, Tacoma Narrows, Turn Rock, and Gordon Island; Figure 4b). No sporophytes developed above 18°C, and significantly fewer sporophytes developed at 18°C compared with cooler temperatures (Figure 4b). Across all populations and nitrogen levels, the average density of sporophytes

was 78% lower at 16°C and 95% lower at 18°C compared to the mean density at cooler temperatures (10–14°C). Sporophyte density was extremely sparse at 16°C for all populations except Turn Rock and Gordon Island (Figure 4b). The production of sporophytes was highest from 10 to 14°C, but populations differed slightly in the temperature of peak production (Figure 4b). Tacoma Narrows, Vashon Island, Lincoln Park, and Edmonds developed the most sporophytes at 10–12°C, Squaxin Island and Gordon Island at 12–14°C, and Turn Rock at 14°C (Figure 4b). The total density of sporophytes also differed significantly across source populations; Tacoma Narrows, Turn Rock, Gordon Island, and Vashon Island produced more sporophytes than Squaxin Island, Lincoln Park, and Edmonds (Figure 4b, Table 2). This trend can be explained partly by the hump-shaped relationship between gametophyte density and sporophyte density (Figure S5 in the Supporting Information), as gametophyte densities from Edmonds may have been too high for successful sporophyte production.

The size of sporophytes also responded significantly to temperature, nitrogen, and source population (mixed-effects model, $p < 0.001$; Table 2). Sporophytes were larger at cooler temperatures (Figure 5), but pairwise tests for temperature within each population were not significant ($p > 0.05$), likely due to the many replicates that did not produce sporophytes at high temperatures. Across all populations that had sporophytes at 18°C, sporophytes were 81% smaller at 18°C compared to cooler temperatures (10–14°C). There were also differences among populations; sporophytes were roughly twice the size at Gordon Island and Turn Rock compared with other populations (post hoc pairwise comparisons, $p < 0.05$; Figure 5).

There were significant effects of nitrogen and the interaction between temperature and nitrogen on the density and size of sporophytes produced (mixed-effects models, $p < 0.001$; Table 2). Within populations, the density of sporophytes responded significantly to nitrogen at certain temperatures, including at 12–16°C at both Turn Rock and Gordon Island, as well as at 10 and 14°C at Tacoma Narrows, and at 12°C at Vashon Island (Figure 4b). In these cases, sporophyte density was significantly greater in the high N treatment. Likewise, sporophytes were generally larger at high N; this trend was significant from 10–14°C at Turn Rock and from 10–16°C at Gordon Island (Figure 5). While the high N treatment led to higher sporophyte densities and larger sporophytes for some populations at cooler temperatures, the high N treatment did not improve sporophyte growth or survival at high temperatures.

DISCUSSION

Rising sea surface temperatures have led to the loss of kelp forest ecosystems around the world (Smale, 2020). In the Salish Sea, where sea surface temperatures are rapidly warming (Amos et al., 2015), bull kelp declines have been most severe in warmer, wave-sheltered areas with low nutrient levels (Berry et al., 2021; Starko

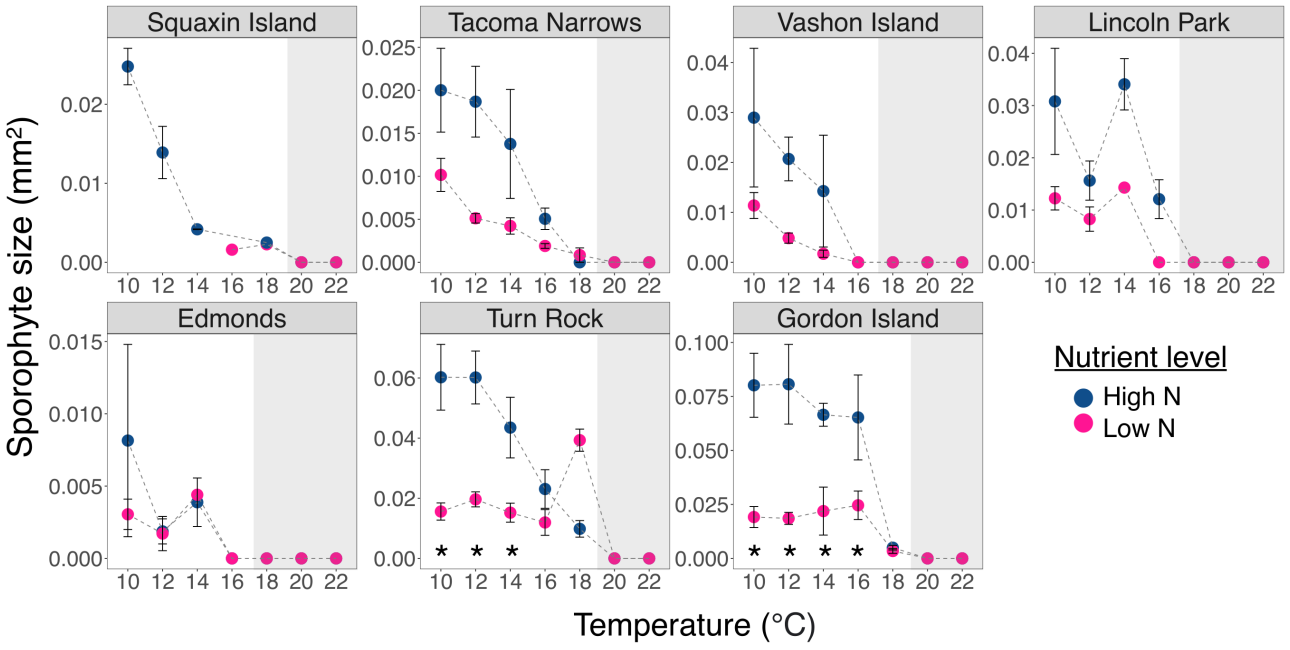

FIGURE 5 Mean (±SE) sporophyte size (mm^2) across temperatures after 32 d of growth. Graphs are separated by source population, with temperature on the *x*-axis and nitrogen treatment indicated by differently shaded points. Within each population, asterisks (*) indicate statistically significant post-hoc comparisons ($p < 0.05$) of mean sporophyte size between nitrogen treatments at a given temperature. The gray panels indicate temperatures at which sporophytes were absent.

et al., 2019, 2022). Studies suggest that elevated nitrogen concentrations can buffer the effect of high temperatures in adult kelp sporophytes (Fernández et al., 2020; Gerard, 1997), but this hypothesis was not supported for microscopic stages of *Nereocystis luetkeana*. Here, we found that the growth and survival of microscopic stages of seven different populations of *N. luetkeana* were more limited by high temperatures than by low nitrogen levels, as additional nitrogen did not improve gametophyte or sporophyte survival at high temperatures. Another study found that gametophyte growth and sporophyte production by *Ecklonia radiata* was limited by high temperatures, with no effect of nitrate concentration (Mabin et al., 2013). Similarly, *N. luetkeana* zoospores from two populations did not germinate at 18°C, regardless of nitrogen levels (Muth et al., 2019). Fernández et al. (2023) found that gametophytes of *Macrocystis pyrifera* were limited above optimum temperatures of 15–17°C despite high nitrogen concentrations. While we found significant interactive effects of temperature and nitrogen on gametophyte growth as well as sporophyte density and size, the effects of nitrogen were limited to a subset of populations and a few temperatures. For example, in four out of seven populations, sporophyte density was significantly greater in the high N treatment, but only at select cool temperatures. Additional nitrogen also enhanced the size of sporophytes, but only for two populations (Turn Rock and Gordon Island) at 16°C and below. We note that our low nitrogen treatment is likely higher than other studies, as our low N media started with seawater containing ~20 μM NO_3. However, after 1 week of growth, low N media had <1 μM NO_3, while the high N media was still N-replete (7 μM NO_3).

While the beneficial effects of nitrogen were limited, the deleterious effects of high temperatures were ubiquitous across populations. Gametophyte densities were highest from 10 to 16°C and significantly lower at 18°C; both 20 and 22°C were lethal regardless of source population or nitrogen treatment. Since all treatments started with the same zoospore density, the 59% decline in gametophyte densities at 18°C likely reflects lower zoospore germination rates rather than mortality, as gametophytes increased in size over time at 18°C (Figure S2). Similarly, another study found that zoospore germination rates from different populations of *Nereocystis luetkeana* from British Columbia were lower at 17.5°C compared with 10 and 15°C (Schiltroth, 2021). Surprisingly, we did see initial zoospore germination at 20 and 22°C from Turn Rock and Gordon Island, but not from other populations, although a few gametophytes germinated from Lincoln Park at 20°C (Figure S2). However, exposure to these temperatures for more than 10 d was lethal. Vadas (1972) also found that gametophytes of *N. luetkeana* germinated and grew at 20°C; however, they did not produce sporophytes, and cells became bleached. Another study found that a very low proportion of *N. luetkeana* zoospores germinated at 21°C (Lind & Konar, 2017). In contrast, Muth et al. (2019) found that *N. luetkeana* zoospores did not germinate at 18°C. We found that *N. luetkeana* gametophytes from all seven populations were able to germinate and continue growing vegetatively at 18°C for up to 6 weeks. Across populations, gametophyte growth rates were highest between 14 and 18°C. Other studies have reported relatively high optimum temperatures for kelp gametophyte growth of 15–18°C (Fernández et al., 2023; Le et al., 2022; Paine et al., 2021).

To complete their annual life cycle, bull kelp gametophytes must undergo sexual reproduction and produce juvenile sporophytes. Although gametophytes were able to grow from 10–18°C, sporophyte production peaked at cooler temperatures of 10–14°C across populations. Our findings corroborate previous studies suggesting that kelp gametophytes have a higher thermal tolerance compared to microscopic sporophytes, potentially acting as microscopic seedbanks that can persist through unfavorable environmental conditions (Martins et al., 2022; Veenhof et al., 2022). In our experiments, the maximum temperature of sporophyte production was 16 or 18°C for all populations, but the density of sporophytes was 78% and 95% lower at these higher temperatures, respectively, suggesting that prolonged exposure of bull kelp gametophytes to temperatures ≥16°C could limit sexual reproduction. The mechanism responsible for the lack of sporophyte production at high temperatures is not known—for example, high temperatures may influence sperm swimming or vitality, pheromone release, or fertilization of the female oogonia. Interestingly, we observed many abnormal and rounded cells on gametophytes at 18°C (Figure S4). Vadas (1972) also noted that at 20°C, "occasionally large cells were produced which may have been aborted oogonia" (p. 198). It is possible that these rounded cells were oogonia that were not fertilized or could not produce sporophytes. Another reason for reproductive failure at 18°C could be low gametophyte densities (Figure S5), as there is a hump-shaped relationship between gametophyte (or zoospore) density and sporophyte production (Reed et al., 1991; Tatsumi et al., 2022).

Populations of *Nereocystis luetkeana* in the Salish Sea displayed differences in genetic diversity via microsatellite markers, and populations in South Puget Sound displayed the lowest allelic richness (Gierke, 2019). Populations with low genetic diversity can be more susceptible to marine heatwaves (Wernberg et al., 2018). Given these genetic differences, we expected to see evidence of local adaptation or differential susceptibility to high temperatures. However, we found no evidence for local adaptation to high temperatures among seven populations from distinct locations within the Salish Sea; gametophytes and microscopic

sporophytes from each population displayed similar thermal response curves and critical upper temperatures for survival and reproduction. Other studies have reported intraspecific differences in thermal tolerance of kelp gametophytes from geographically separated populations (Becheler et al., 2022; Martins et al., 2020; Schimpf et al., 2022; Strasser et al., 2022). However, populations in these studies were separated by ~500 to >2500 km, while the two most distant populations in this study (Gordon Island and Squaxin Island) are separated by only ~200 km, and most populations are less than 50 km apart (Figure 1). Another study found that two populations of *N. luetkeana* from California and British Columbia both germinated at 12°C but not at 18°C, showing a lack of local adaptation (Muth et al., 2019), although temperatures between the two extremes were not tested. The lack of local adaptation in gametophyte thermal tolerances across populations could be due to sufficient dispersal of zoospores between populations (gene flow) or variability in environmental selection, or it could be constrained by a lack of genetic variation (Takahashi et al., 2016). Adaptation to high temperatures is only possible when genetic diversity is directly linked to variation in thermal tolerance (Alsuwaiyan et al., 2021). Future studies might combine genetic sequencing and environmental temperature monitoring to determine whether there is any adaptive genetic diversity among populations of bull kelp in the Salish Sea that may allow them to perform better at elevated temperatures, similar to Vranken et al. (2021).

Although bull kelp populations had similar thermal response curves, populations differed in the magnitudes of gametophyte and sporophyte densities. For example, gametophyte densities from the Edmonds population were twice as high as other populations despite starting with the same zoospore density. This difference could reflect differential zoospore viability or germination rates among populations. Germination rates of *Nereocystis luetkeana* zoospores can be highly variable both within and among populations throughout the reproductive season (Schiltroth, 2021), likely contributing to variation in gametophyte densities between populations. Logistical constraints led us to split the seven populations into two experiments in July and August (Table 1), and two of the populations that produced the highest sporophyte densities (Turn Rock and Gordon Island) were grown in August, possibly reflecting differences in the seasonality of kelp reproduction (Tatsumi et al., 2022).

We found that prolonged exposure of bull kelp gametophytes to temperatures ≥16–18°C limited gametophyte survival and sporophyte production, and in situ bottom temperature sensors revealed that these critical thermal limits were reached in the late summer at multiple sites. However, gametophyte survival at high temperatures depends on exposure time (Martins et al., 2020; tom Dieck, 1993), and our study investigated long-term exposure to elevated temperatures. At Squaxin Island, daily maximum temperatures exceeded 16°C for 50 consecutive days from July 25 to Sept. 12, 2022, which is longer than the duration of our experiments. It is likely that elevated bottom temperatures at Squaxin Island have contributed to kelp forest declines at that site, but other factors such as grazing by kelp crabs (*Pugettia producta*) or competition from the invasive species *Sargassum muticum* are also major stressors (Berry et al., 2021). Elevated temperatures are often punctuated by periods with cooler water, as they were at Gordon Island and Cherry Point (Figure 2). Warm periods at these sites were likely due to variation in the warm outflow from the Fraser River (Lowe et al., 2016). Future studies could examine the effects of shorter and variable duration marine heatwaves, followed by cool recovery periods, to determine the ability of microscopic stages of bull kelp to recover from warming events.

Kelp persistence at the warmest sites likely depends on the duration of elevated temperatures and their coincidence with the phenology of kelp reproduction and growth. The annual recruitment of bull kelp sporophytes in the Salish Sea begins in early March (Maxell & Miller, 1996), and sporophytes usually reach the surface by late spring, although juvenile sporophyte recruitment can continue throughout the year (Dobkowski et al., 2019). Even at the warmest sites, bottom temperatures in March and April remained well below 12°C, which would allow juvenile sporophytes to develop before temperatures rise. Interestingly, in northern California, winter sea surface temperatures predicted bull kelp canopy cover, and the authors concluded that nutrient limitation associated with warmer water may explain this trend (García-Reyes et al., 2022). However, we found high temperatures were much more limiting than nitrogen levels for gametophyte and juvenile sporophyte growth and survival. Elevated summer temperatures can impact the late recruitment of juvenile bull kelp, as recruitment can continue throughout the summer into early fall (Maxell & Miller, 1996) and even winter (Dobkowski et al., 2019). Adult bull kelp sporophytes become reproductive and release zoospores primarily from late spring throughout the summer, but fertility peaks in July (Maxell & Miller, 1996). Gametophytes that germinate on the benthos during the peak reproductive season are exposed to the warmest seawater temperatures of the year, and temperatures ≥18°C could limit gametophyte survival. Two sites, Squaxin Island and Cherry Point, had daily maximum temperatures that exceeded 18°C, but only for a maximum of three consecutive days (out of the nine total days) at Cherry Point. Finally, the development of zoospores within reproductive sori could also be impacted by elevated seawater temperatures. Elevated temperatures increased zoospore release rates of

Macrocystis pyrifera but decreased zoospore settlement, germination, and survivorship (Le et al., 2022). In a short-term heatwave experiment, reproductive blades of *Nereocystis luetkeana* that were incubated at 18, 20, and 21°C for 3.75 days produced zoospores that germinated at cooler temperatures and developed into gametophytes and juvenile sporophytes (Vliet et al., 2022). High temperatures clearly limit the reproductive cycle of bull kelp, but we need a better understanding of how the timing and intensity of marine heatwaves impacts gametophyte and juvenile sporophyte survival in the field.

In a warming global ocean, it is critical to determine the thermal tolerance of foundation species such as bull kelp so that we can model the future effects of climate change on kelp forest ecosystems. Although we cannot lower seawater temperatures in the Salish Sea without significant efforts to mitigate climate change on a global scale, we can use this information to prioritize management and conservation actions (Hollarsmith et al., 2022). We observed that high nitrogen concentrations did not enhance the survival of microscopic stages of bull kelp that were exposed to elevated temperatures. This suggests that management actions that increase nutrient inputs are not likely to enhance bull kelp survival or recovery in the face of rising temperatures. In addition, nutrient inputs are generally associated with kelp losses through complex interactions including light reduction and growth of competitive turf algae (Filbee-Dexter & Wernberg, 2018; Tait et al., 2021). We noted that prolonged exposure to temperatures ≥16–18°C could limit gametophyte survival and sporophyte production across all populations. Continued monitoring of temperatures within kelp forests across the Salish Sea is important to identify sites that are experiencing elevated temperatures, making them susceptible to declines associated with ocean warming. In addition, bottom and surface temperature data can support identification of suitable sites for kelp forest restoration. Spatial planning for restoration sites should consider bottom and surface temperatures in addition to other habitat characteristics.

AUTHOR CONTRIBUTIONS

Brooke L Weigel: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (equal); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Sadie L Small:** Data curation (equal); investigation (equal); methodology (supporting); writing – review and editing (equal). **Helen D Berry:** Conceptualization (supporting); funding acquisition (equal); methodology (supporting); writing – review and editing (equal). **Megan Dethier:** Conceptualization (supporting); funding acquisition (lead); methodology (supporting); supervision (lead); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

Environmental temperature data generated during this study are available on the Figshare data repository: https://doi.org/10.6084/m9.figshare.22695658.v3. The other datasets are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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

Figure S1. Total sporophyte density (sporophytes per cm^2) across different zoospore densities at two different temperatures (10 and 12°C). This assay was performed before the experiments to determine the optimal density of zoospores for sporophyte production.

Figure S2. Growth curves showing the mean (±SE) gametophyte size (mm^2) over the course of the experiment, from day 10 to day 31. Graphs are separated by temperature, indicated at the top in gray panels, and points are colored by population. Growth was positive for all temperatures except 20 and 22°C. Gametophytes from a few populations germinated at 20 and 22°C, but they were dead by day 17.

Figure S3. Gametophyte growth rate vs. gametophyte density for all replicate wells, with points colored according to (a) growth temperatures, and (b) source populations. The trend line represents a quadratic polynomial regression fit (LOESS method).

Figure S4. Example microscope photos of gametophytes grown at 18°C that developed rounded cells. Arrows point to example rounded cells, of which there are many, on each gametophyte.

Figure S5. Sporophyte density vs. gametophyte density for all replicate wells, with points colored according to (a) growth temperatures, and (b) source populations. The trend line represents a quadratic polynomial regression fit (LOESS method).

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