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Thermal priming of dehisced *Posidonia* seed does not increase thermal tolerance in establishing seedlings

Theme: Benthic Habitats and Communities
WAMSI Westport Marine Science Program



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ABOUT THE MARINE SCIENCE PROGRAM

The WAMSI Westport Marine Science Program (WWMSP) is a \$13.5 million body of marine research funded by the WA Government. The aims of the WWMSP are to increase knowledge of Cockburn Sound in areas that will inform the environmental impact assessment of the proposed Westport development and help to manage this important and heavily used marine area into the future. Westport is the State Government's program to move container trade from Fremantle to Kwinana, and includes a new container port and associated freight, road and rail, and logistics. The WWMSP comprises more than 30 research projects in the biological, physical and social sciences that are focused on the Cockburn Sound area. They are being delivered by more than 100 scientists from the WAMSI partnership and other organisations.

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DATA

Finalised datasets will be released as open data, and data and/or metadata will be discoverable through Data WA and the Shared Land Information Platform (SLIP).

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FRONT COVER IMAGE

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Front cover image: Seagrass (*Posidonia australis*) in Cockburn Sound. Photo courtesy of: Rachel Austin (The University of Western Australia).

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The WAMSI Westport Marine Science Program is a \$13.5 million body of research that is designed to fill knowledge gaps relating to the Cockburn Sound region. It was developed with the objectives of improving the capacity to avoid, mitigate and offset environmental impacts of the proposed Westport container port development and increase the WA Government's ability to manage other pressures acting on Cockburn Sound into the future. Funding for the program has been provided by Westport (through the Department of Transport) and the science projects are being delivered by the Western Australian Marine Science Institution.

Thermal priming of dehisced *Posidonia* seed does not increase thermal tolerance in establishing seedlings

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Theme 2, Project 2.2: Pressure-response relationships, building resilience and future proofing seagrass meadows

Executive Summary

Under a warming climate, temperate seagrasses are under threat from rising seawater temperatures and the increased frequency and intensity of marine heatwaves. Furthermore, seagrass meadows growing within coastal zones near anthropogenic activities may be further subjected to impacts from low light and burial stress associated with dredging. Indeed, port development and associated dredging operations are currently proposed in Cockburn Sound, Western Australia. Strategies to enhance the resilience of seagrasses to heat related stress was recently conducted in *Posidonia oceanica* seedlings in the Mediterranean (Pazzaglia et al 2022). Here in Western Australia, and in light of the proposed Westport dredging operations in Cockburn Sound, we adopted this novel approach and conducted a mesocosm experiment to test whether the thermal priming of *Posidonia australis* seeds led to enhanced survival and growth in seedlings under elevated seawater temperatures comparable to a MHW scenario, in order to inform restoration practices and identify if this was an option to build resilience of restored seagrass to marine heatwaves.

Mature *P. australis* fruit were collected from Woodman Point, Cockburn Sound, Western Australia in early December 2023, as part of the 'Seeds for Snapper' program operated by OzFish. Once collected, fruit were maintained in continuously flowing seawater tanks at ambient temperature (21 °C) until dehiscence. Seed(lings) were collected and transported to Edith Cowan University, where a mesocosm experiment was implemented. A fully crossed experimental design was employed with a pre-treatment (no thermal priming of seeds (unprimed control) or thermal priming to 26 °C (+ 5 °C)). Seedlings then remained at ambient temperature (control: 22 °C) or were exposed to an elevated temperature to simulate a marine heatwave (28 °C). Four replicated glass aquaria were used for each treatment (Unprimed-Ambient, Unprimed-Elevated, Primed-Ambient, and Primed-Elevated). 256 seedlings were monitored for survival and growth (leaf number, maximum leaf length, necrosis) *in situ* at six timepoints throughout the experiment.

The key outcomes from this research were:

- Seedling survival after three months (T12) was $\geq 80\%$ in all treatments and significantly fewer seedlings survived the elevated temperature treatment (28 °C) regardless of priming pre-treatment. There was a decline in survival of all seedlings during the year-long experiment, down to 12-31 % but this is much higher than what is observed under natural settings (1 %).
- There were no positive effects of priming. Leaf growth and root development was reduced under marine heatwave conditions, regardless of the priming pre-treatment, despite the method showing promise to build thermal tolerance in mature seagrass plants.
- At the photo-physiological level, there was a significant interaction between the priming and heatwave treatment for photosynthetic yield. The yield of unprimed seedlings was higher than primed seedlings under heatwave conditions, not ambient conditions. Inferring that unprimed seedlings were performing better in MHW conditions compared to ambient conditions at the physiological level.
- Seed priming using these conditions should not be applied to *P. australis* seed(lings) at the current time. It is recommended to explore other priming conditions. Currently the most promising resilience building options are to trial restoration using seedlings from environments where plants may be warm-adapted (see Whale et al., in review).

1 Introduction

Climate change is the single largest threat to global biodiversity and ecosystem function (Grimm et al., 2013; Barnosky et al., 2011; IPBES, 2019). It has contributed to species and population-level extinctions as well as changes in species distributions and ecological interactions (Pecl et al., 2017; Vergés et al., 2019, Enright et al., 2015). One of the key drivers of climate change is global warming with the warmest years on record occurring in recent years (Abram et al., 2016; NOAA Coral Reef Watch, 2022; Skirving et al., 2020; Stocker et al., 2013). Both gradual warming and extreme climatic events, such as heatwaves, are major disruptors to ecosystems with significant ecological impacts documented from marine heatwaves (MHWs) in a range of habitats and species (Wernberg et al., 2024, Spalding and Brown, 2015; Smale et al., 2019; Frölicher et al., 2018, Kendrick et al., 2019). Species must either adapt with environmental changes, migrate to suitable refugia, or risk extinction (Aitken et al., 2008; Allen et al., 2010; Davis and Shaw, 2001).

Building resilience in natural systems is an active research field that aims to improve conservation outcomes through actively trying to mitigate the effects of climate change by minimising population declines or loss in ecosystem function (Correia et al., 2021; Janni et al., 2020., Liu et al., 2022). There are a range of approaches which require different levels of understanding and intervention. For example, maintaining genetic diversity and gene flow through assisted migration (e.g. Sgrò et al., 2011; Aiken and Whitlock, 2013, Prober et al., 2015), breeding, assisted evolution, genetic engineering and biotechnology approaches (e.g. van Oppen et al., 2015) or priming (Liu et al., 2022). Resilience building is developing rapidly in coral reef ecosystems where the impacts from warming are well documented (Sully et al., 2019), the potential for adaptation has been identified (e.g. Palacio-Castro et al., 2023) and multiple techniques such as assisted migration and evolution are being trialled and implemented (van Oppen et al., 2015, Bay et al., 2023). Priming is one of the resilience building tools that has received traction in the seagrass community.

Priming (also known as acclimating, conditioning, or hardening) individuals to novel or stressful conditions can enhance the tolerance or productivity in less favourable conditions (Costa e Silva et al., 2021; McDowell et al., 2008; Wang et al., 2017). This is because all species have an environmental niche within which metabolism functions at its optimum, and outside of this niche there is stress and decline in performance (Donoghue and Edwards, 2014; Wasof et al., 2013). From the perspective of temperature, the risk of local extinction increases when temperature exceeds this optimal thermal range. This is especially significant for adults of benthic marine organisms that have limited ability to migrate (Hargreaves et al., 2015; Rehm et al., 2015, O’Sullivan et al., 2016). Greater exposure of individuals to a particular environmental stress (priming), such as heat, may reduce sensitivity to subsequent exposures and therefore they become more resilient into the future. Priming can be applied across different life history stages, although it is most common in adults or seedlings. The theory of priming as a conservation tool is that it stimulates epigenetic modifications that can potentially alter gene expression and mitigate the effects of stress (Bossdorf et al., 2008). Much of the research conducted to date, has explored the effect of priming to enhance resilience in terrestrial trees (Ahrens et al., 2021; Ahrens et al., 2022) and crop species (Robison et al., 2017; Tombesi et al., 2018; Wang et al., 2019) and there have been a few studies on seagrasses (e.g. *Posidonia* and *Zostera*) in the Mediterranean and Australia (DuBois et al., 2020; Nguyen et al., 2020; Pazzaglia et al., 2022).

Seagrass meadows are some of the most diverse ecosystems globally; however, there have been large declines in cover, extent or density and subsequent loss of ecosystem function (Waycott et al., 2009; Dunic et al., 2021) due to a range of human and natural activities (Turschwell et al., 2021; Erftemeijer and Lewis, 2006; Sagerman et al., 2020), as well as climate change (Pruckner et al., 2022; Strydom et al., 2020; Thomson et al., 2014). MHWs have significantly impacted seagrasses in temperate and subtropical regions where meadows have been exposed to extreme temperatures for long periods of time, indicating a lack of resistance to heat stress (Marbà and Duarte, 2010, Hammer et al., 2018, Fraser et al., 2014; Strydom et al., 2020, Nowicki et al., 2017). Considering MHWs are predicted to

increase in frequency, intensity, and duration (Cai et al., 2014; Evans et al., 2014; IPCC, 2018) and seagrass species generally have wide distributions, over large thermal gradients (Short et al., 2007), there is the potential for local adaptation to temperature in populations (e.g. Whale et al, in review) with associated differences in thermal optima (e.g. Said et al., in review). Bennett et al. (2022) also demonstrated experimentally that *Posidonia oceanica* populations at the warm-range edge were less sensitive to thermal stress (> 29 °C) than populations experiencing naturally cooler water temperatures. This makes seagrass species with broad distributional ranges ideal candidates to trial priming as a resilience building strategy, particularly considering that some seagrasses could experience upper thermal limits consistently within 50 years under an extreme greenhouse gas emission scenario (Marbá et al., 2022). Indeed, sea surface temperature (SST) predictions to the year 2100 indicated that the annual mean rate of SST increase was 0.015 and 0.03°C per annum, under Shared Socioeconomic pathway scenario 2 (SSP2) and scenario 5 (SSP5), respectively. This is predicted to increase the mean SST by 1.27°C and 3.31°C by 2100 under SSP2 and SSP5, respectively, in Cockburn Sound (Pattiaratchi et al., 2024). Thermal priming (*ex situ*) has been examined in adults and seedlings of a few seagrass species recently (DuBois et al., 2020; Nguyen et al., 2020; Jueterbock et al., 2021; Pazzaglia et al., 2022). Temperate seagrass genera (*Posidonia* and *Zostera*) have shown enhanced photosynthetic capacity (Nguyen et al., 2020; Pazzaglia et al., 2022) and leaf growth (Dubois et al., 2020; Nguyen et al., 2020) following priming when re-exposed to elevated temperatures with evidence they may be linked to epigenetic modifications (Jueterbock et al., 2020).

Posidonia australis is one of the most ecologically valuable habitat-forming species in temperate Australian waters (Middleton et al., 1984). It is a persistent, slow growing species that can produce large quantities of buoyant fruit annually. Significant loss of *Posidonia* dominated meadows in Australia have been associated with anthropogenic activities (e.g. Cockburn Sound, Kendrick et al., 2002; New South Wales estuaries, Glasby and West, 2018) as well as extreme climate events (e.g. Shark Bay, Strydom et al., 2020). Programs such as ‘Seeds for Snapper’ and ‘Operation Posidonia’ (Sinclair et al., 2021) use both seeds and adult plants to restore damaged meadows. Therefore, this is an ideal model species to assess the potential for priming to build thermal resilience into seagrass restoration programs.

We conducted a mesocosm experiment to test whether thermal priming of seeds could be used to build resilience to MHWs for the temperate seagrass *Posidonia australis*. We propose that if i) primed seedlings perform better under MHW conditions and are not negatively impacted under non-MHW conditions and if ii) unprimed seedlings perform worse at elevated temperatures, then priming could be considered a valuable resilience building strategy to be incorporated into seed-based restoration programs.

2 Materials and Methods

2.1 Seagrass Collection

Posidonia australis Hook.f. is a long-lived seagrass species endemic to the temperate waters of Australia, with a widespread distribution ranging from Shark Bay, Western Australia (WA) where thermal optimum measured 30.5°C, and 27.4°C in Perth populations (Said et al in review), to Wallis Lake in central New South Wales, Australia (Sinclair et al., 2014). As a marine flowering plant, it can grow in continuous meadows from the shoreline to an approximate depth of 15 metres favouring the more sheltered habitats (Cambridge and Kuo 1979; Carruthers et al., 2007). It is a foundation species of coastal ecosystems providing key ecosystem services that include carbon sequestration, nutrient cycling, and sediment stabilisation mitigating coastal erosion (Fourqurean et al., 2012; Nordlund et al., 2016; Orth et al., 2006). Mature fruits of this species are released in the late austral spring/early summer (October-December), are buoyant, and once on the sea surface are subjected to the local surface currents and windage (Ruiz-Montoya et al., 2012). The buoyant fruit can remain afloat for up to a week before releasing a single, large, negatively buoyant seed (Ruiz-Montoya et al., 2015). The

seeds are often referred to as ‘direct developers’, as they usually have an emerging plumule or young leaves when dehisced (i.e. there is no dormancy phase).

Mature *P. australis* fruit were collected immediately prior to dehiscence by snorkelers and SCUBA divers as part of the Seeds for Snapper program in early summer (December) of 2022 from meadows off Woodman Point, Perth, Western Australia (-32.13291, 115.74614, GDA 1994), representing a northern Cockburn Sound cohort (Sinclair et al., 2014). Maturity was monitored over a 4-week period before collection to ensure the most mature fruit were collected. Once collected, fruit were maintained in continuously flowing seawater at ambient temperature (21 °C) until dehiscence in a 2,500 litre flow-through tank (as described in the Seeds for Snapper case study; Sinclair et al., 2021). Seedlings were then transported in sea water to the mesocosm facility at Edith Cowan University, Joondalup, WA.

2.2 Experimental Design

A fully crossed design was employed with either pre-treatment option of thermal priming or no thermal priming of seeds (control). following by a MHW phase, then a recovery phase (Figure 1A). For each treatment regime (Unprimed-Ambient, Unprimed-Elevated, Primed-Ambient, Primed-Elevated), there were four replicate glass aquaria tanks. The pre-treatment period was conducted over ten days, and then the heatwave treatment was imposed for 80 days. After this all tanks were returned to ambient temperature and the survival post heatwave assessed for 257 days. Measurements were conducted at multiple timeframes over the heatwave treatment, and the frequency varied depending on the measures: at the start of the heatwave treatment (T0), after 1-week (T1), 2-weeks (T2), 4-weeks (T4), 8-weeks (T8), and 12-weeks (T12) during the heatwave treatment and then 3 monthly after the heatwave treatment was stopped. The pre-treatment occurred between 7th December to 16th December 2022, the heatwave between 23rd December 2022 to 17th March 2023, and the recovery/long-term monitoring from 17th March to 28th November 2023.

All seedlings were planted onto washed Gingin quartz sediment (Soils Ain't Soils, Neerabup, WA) within 2.5 litre plant pots with addition of 0.5 g of dried seagrass wrack 2 cm below the surface to provide a slow-release nutrient supply (Lavery et al., 2013; Statton et al., 2013). Four seedlings were planted per pot separated by a minimum distance of 2 cm. Sixty-four pots were placed into six 500 litre tanks, each connected to a 100 L sump tank fitted with a PondMax EV 2910 3000 litre pump (Pondshop, Midvale, WA) and Pentek 25 micron filter (Clarence Water Filters, Yamba, WA). Water temperatures were maintained using a TK-2000 heater-chiller unit (TECO, Fornace Zarattini, Italy, accuracy ± 1 °C). During the pre-treatment phase seedlings were split into two treatment groups: a control group which remained at ambient water temperature (21 °C) and a thermal priming treatment at 26 °C. This thermal priming temperature of 26 °C was selected to represent a Category I marine heatwave event in the region from which seeds were collected (Hobday et al., 2018). Seedlings remained within the thermal optimum temperature for mature *P. australis* plants (27.4 °C, Said et al., *under review*) from the same location. No thermal optima exist for seedlings. Water temperatures were increased at a rate of 1 °C per day until reaching 26 °C, maintained for 24 hours and then decreased at a rate of 1 °C per day to ambient for a total of 10 days priming above ambient temperature (Figure S1).

For the heatwave phase, pots were then transferred to the Edith Cowan University aquarium facility and placed across 16 50 L glass aquaria tanks, and allowed to acclimate for seven days at 21 °C. Each glass aquaria were connected to a 60 L sump with four pots per tank. Following acclimation, seedlings were split evenly into two further groups; one half of the unprimed seedling pots and half of the primed seedling pots (n = 64 seedlings across 16 pots per treatment) remained at ambient seawater temperatures (now 22 °C, consistent with historic average water temperatures in Cockburn Sound for late December-early January (NOAA Coral Reef Watch, 2022; Skirving et al., 2020) and the other half of unprimed and primed seedlings were exposed to a more severe and prolonged heatwave of 28 °C for three months (Category IV; Hobday et al., 2018). The temperature of 28 °C was selected to reflect

an extreme marine heatwave experienced by *P. australis* meadows in Shark Bay (Fraser et al., 2014; Wernberg et al., 2013) and central Cockburn Sound (Rose et al., 2012) during the summer of 2010-2011. This is just above the optimum temperature for adult *P. australis* plants in Perth (27.4°C). All water temperature changes occurred at a rate of 1 °C per day. Each ambient tank was fitted with a 3000 L per hour PondMax EV 2910 pump connected to a TK-150 chiller unit (TECO) and elevated tanks fitted with ≥ 750 L Aqua Bee pumps (ABS Technologies, Littlehampton South Australia, Australia) and a temperature-adjustable TH 500 titanium heater (Aqua One, Sydney, Australia). All tanks were either fitted with a Grow Sun LED Light or a GR-08-V1 Grow Series Light (Marintech). Photoperiod was set to 14:10 hour ratio consistent with summer daylight hours for Perth, WA. Light intensity was measured using a micro PAR light meter (Insitu Marine, Bibra Lake, WA) with each tank receiving above-saturation light of 175 - 235 PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$). Water temperatures, salinity, and dissolved oxygen were measured daily using a YSI professional plus probe (YSI, Yellow Springs, Ohio, USA) (Figures S2 – S4). Water temperatures remained within <0.25 °C of the Elevated treatments and there was slightly more variation in the Ambient treatments (<1.0 °C). Salinity was more variable but generally between 35 – 37 ppt, and slightly higher in the Elevated treatments. Natural seawater was locally sourced from Hillarys Marina, and part-changed weekly in all tanks. After the heatwave treatment, water temperatures for all aquaria were maintained at 22 °C for three months following the cessation of the simulated prolonged heatwave (March to June 2023) and then maintained at an ambient winter temperature of 18 °C for the remaining six months (June to December, 2023).

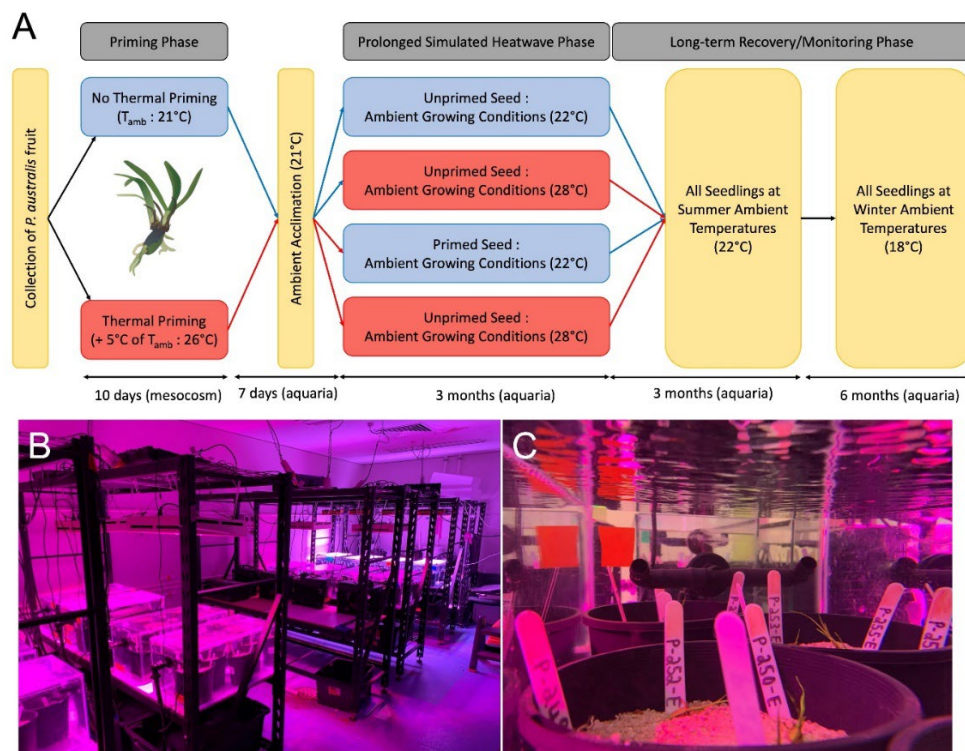


Figure 1: (A) Experimental design for the resilience building experiment of *P. australis* seeds. (B) Photo of the randomised tank set-up in the aquarium room, and (C) Close-up within one of the tanks within the room set-up.

2.3 Leaf Growth, Leaf Damage and Chlorophyll Fluorescence

We monitored maximum leaf length and leaf number *in situ* for all 256 seedlings at multiple timepoints throughout the experiment (T0, T1, T2, T4, T8, T12, T24, T36, and T48, Table 1). Maximum leaf length was measured using a ruler (± 0.1 cm accuracy) from the base of the plumule to the tip of the longest leaf. From this data, relative growth rate (mm d^{-1}) was estimated from the maximum leaf length;

standard measures used in published *P. australis* seedling experiments (Strydom et al., 2018) and other seagrass thermal-priming work (Dubois et al., 2020; Nguyen et al., 2020). A visual assessment of leaf necrosis or tissue damage was conducted to quantify any physical damage to the seedlings. Leaf damage was characterised as a change in colour from green to brown or black colouration; see Ferretto et al. (2023). All seedlings were assessed for leaf damage from T2. Leaf damage was quantified as the estimated percentage of necrosis observed across the full set of leaves for each seedling.

Table 1: Traits measured throughout the priming experiment. T0 = end of the acclimation period before the simulated marine heatwave (MHW) of 28 °C; T1 = 1 week after the start of the MHW; T2 = 2 weeks into the MHW; T4 = 4 weeks/1 month; T8 = 8 weeks/2 months; T12 = 12 weeks/3 months; T24 = 24 weeks since the experiment started and 12 weeks/3 months after the end of the MHW; T36 = 36 weeks since the experiment started and 24 weeks/6 months after the end of the MHW; and T48 = 48 weeks since the experiment started and 36 weeks/9 months after the end of the MHW. ☐ = trait measurement collected, if no tick = trait was not collected at that timepoint.

Trait (units)	Timepoint										
	T0	Heatwave Phase							Post-Heatwave Phase		
		T1	T2	T4	T6	T8	T10	T12	T24	T36	T48
Leaf Number (n)	☐	☐	☐	☐			☐		☐	☐	☐
Maximum Leaf Length (mm)	☐	☐	☐	☐			☐		☐	☐	☐
Relative Growth Rate of Maximum Leaf Length (mm day ⁻¹)		☐	☐	☐			☐		☐		
Photosynthetic Yield of Photosystem II			☐	☐	☐	☐	☐	☐			
Number of Leaves with Necrosis (n)			☐	☐			☐		☐	☐	☐
Number of Roots (n)								☐			
Maximum Root Length (mm)								☐			
Leaf Biomass (g)								☐			
Seed Biomass (g)								☐			
Root Biomass (g)								☐			
Total Biomass (g)								☐			
Leaf to Root Ratio								☐			
Seedling Survival (n)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

The photophysiological response of a subset of the seedlings was determined using a Waltz™ pulse amplitude modulation (PAM) fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Repeated dynamic measurements of maximum quantum yield (F_v/F_m) of photosystem II (PSII) were conducted on two randomly selected seedlings from two pots per tank per treatment every two weeks from timepoint T2 to T12. Measurements could not be taken prior to T2 as the leaves were not large enough to be able to take a measure. All measurements of F_v/F_m commenced at 08:30 am after all seedlings had been night-adapted for 8 hours and before the start of the daily light cycle when the lights were turned on. The LED fibre optic was gently placed onto the surface of a lower portion of a newly-developed leaf and supported in place by the user from the leaf surface.

2.4 Above- and Below-ground Biomass

After three months (T12), one randomly determined pot of four seedlings per tank per treatment was removed from the aquarium and the seedlings harvested (i.e. 16 seedlings per treatment). Each seedling was gently removed from the sediment to ensure all (primary and lateral) roots remained intact. Seedlings were rinsed in fresh water, dab-dried with a paper towel to remove excess water and immediately weighed on a 3-decimal analytical balance. Seedlings were then individually photographed before counting the number of roots and measuring the primary (longest) root using a pair of analog callipers (accuracy ± 0.05 mm). Seedlings were then placed into a paper bag and oven dried at 60 °C for four days. The dried material was then separated into three organ types; leaves, root, and seed to measure biomass and weighed on a 3-decimal analytical balance.

2.5 Seedling Survival up to 12 Months

Seedling survival was determined for all treatments throughout the simulated heatwave and at the end of the 3-month treatment (T12) and then every 3 months up to 12 months post heatwave treatment (T24, T36, and T48). Seedling mortality was determined based on visual inspection of the following criteria: the seedling had grown mould (and would be removed), the aboveground (all leaves) were wholly (100 %) necrotic, and/or the seedling possessed zero leaves. Seedlings were determined to be alive if they contained a minimum of one green leaf (capable of photosynthesising).

2.6 Statistical Analyses

All analyses were performed in R (R Core Team, 2019). A Levene's test was performed to check homogeneity of variance and a Shapiro-Wilk test to determine normality of the data. Two-way analysis of variance (ANOVA) was then used to determine significant differences in plant response variables between treatment regimes at three separate time points, after the pre-treatment phase and before the heatwave treatment was imposed (T0), after three months of the heatwave treatment (T12) and then at the end of the post-heatwave (T48). Data were analysed by 2-way ANOVA following log- or rank-transformation of the data where parametric assumptions of normality were not met. When multiple measures were collected from the same tank, values were averaged by pot. A Tukey HSD *post hoc* test was conducted when significant differences were detected between treatment (priming versus non-priming) and temperature (ambient versus elevated) or their interactions.

3 Results

3.1 Post Thermal Priming

Following the thermal priming there were no significant differences in the number of leaves ($p = 0.967$) or maximum leaf length ($p = 0.335$) between the primed and non-primed seeds (Figure 2).

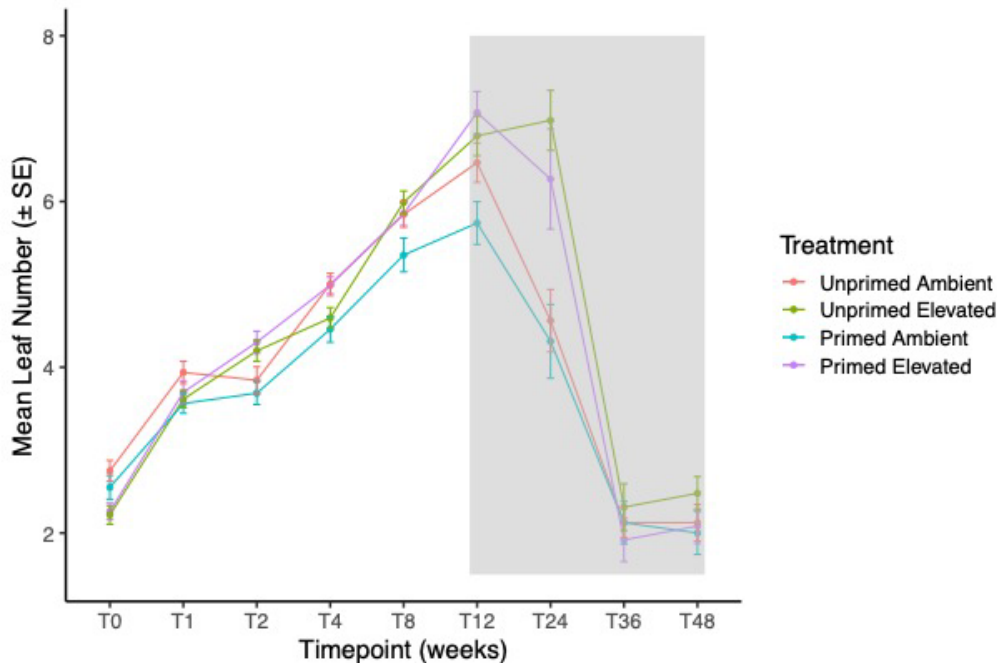


Figure 2: Mean number of leaves for seedlings of each treatment through the experiment. Grey shaded area represents the post-heatwave long-term monitoring phase. Number of seedlings per treatment from T0 to T12, $n = 32$ and from T24 to T48, $n = 16$.

3.2 Heatwave Effects

3.2.1 Survival, Morphology and Growth

There was high survival ($> 80\%$) over the heatwave treatment but at the end of the treatment, survival was higher for seedlings grown under ambient conditions (both unprimed and primed, 96.9%) compared to heatwave conditions (unprimed 81.2% , primed 87.5% , p -value = 0.007), regardless of whether they had been primed or not (Figure 3). All seedlings that survived grew with both the number of leaves and leaf length increasing over time, ranging from 2 to a maximum of 10 leaves (mean maximum of 7, Figure 2). There was an interesting dynamic where over time unprimed seedlings in ambient conditions had more, but shorter leaves compared to the unprimed seedlings in heatwave conditions after one month (T4) e.g. 5 vs 4 leaves of 52 vs 62 mm mean length of longest leaf (Figure 4). The morphology of primed seedlings were more similar after one month and were intermediate to the unprimed treatments. By the end of the heatwave treatment this pattern switched and primed seedlings under heatwave conditions had more, but shorter leaves compared to the primed seedlings under ambient condition e.g. 7 vs 5 leaves of 75 vs 85 mm (Figures 4, 5A, 5B), and the unprimed seedlings were intermediate to this.

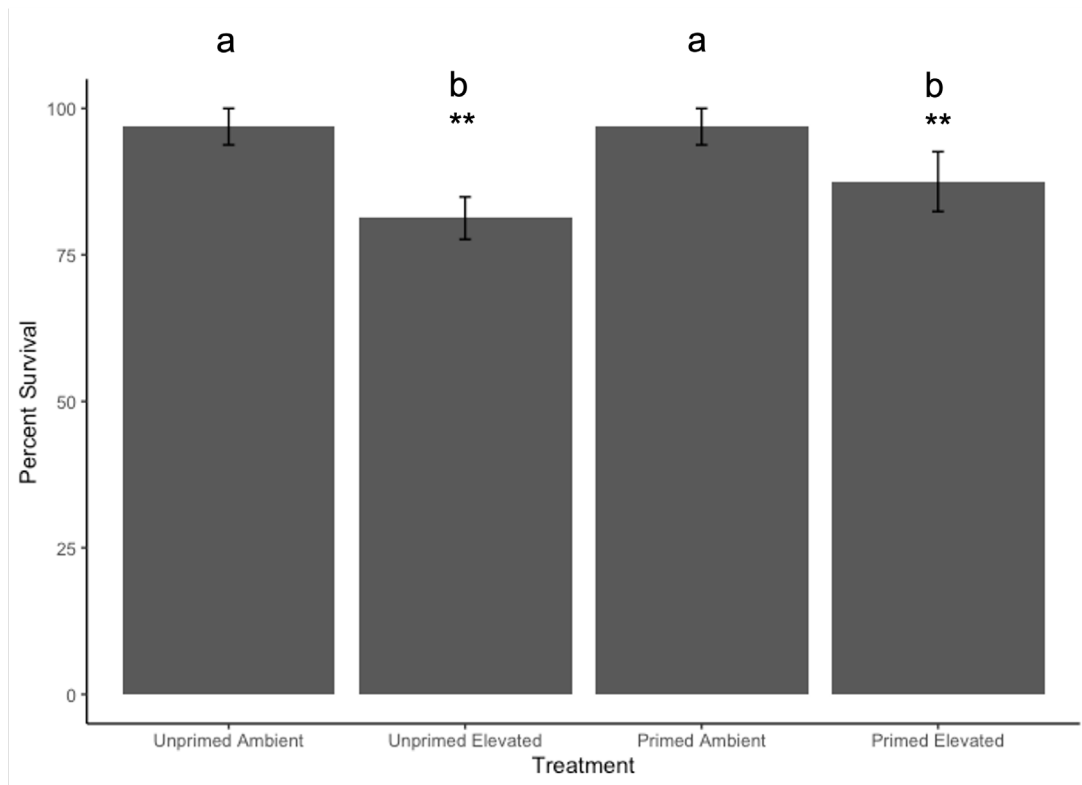


Figure 3: Percent seedling survival (\pm standard error) for all treatments following the 3-month heatwave treatment. **, $p < 0.01$.

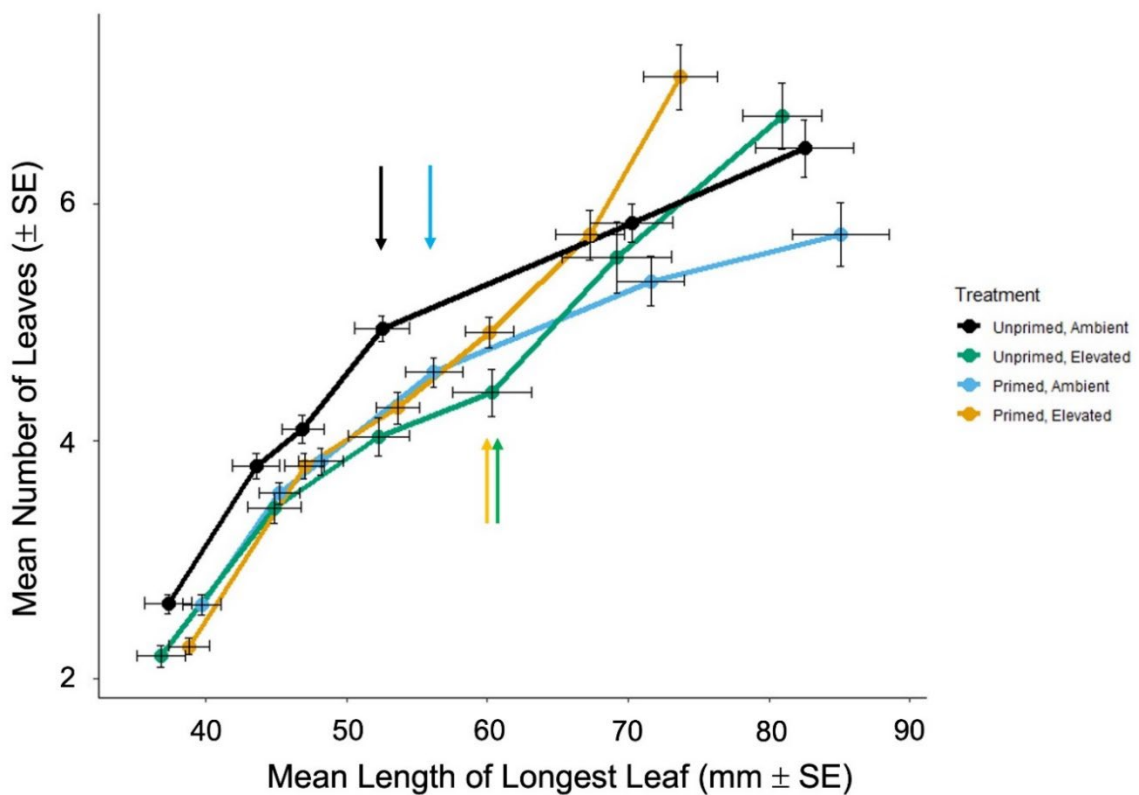


Figure 4: Relationship between mean number of leaves and mean length of the longest leaf (\pm standard error) across all treatments during the 3-month simulated heatwave scenario. Coloured arrows denote the measurements taken 1-month (T4) after implementation of the heatwave scenario.

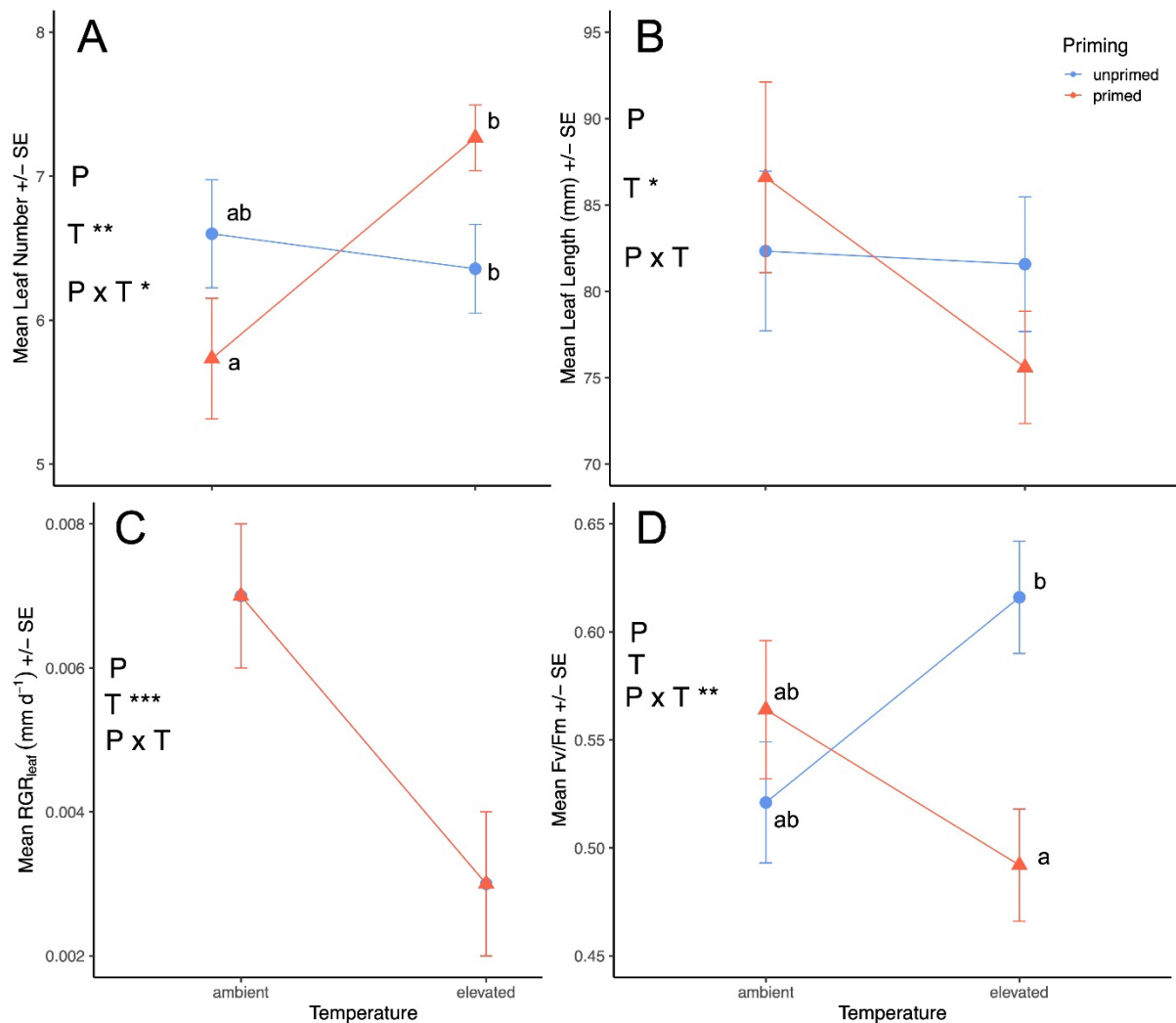


Figure 5: Trait responses for *P. australis* seedlings for each treatment at the end of the 3-month heatwave (T12) for: (A) mean leaf number; (B) mean maximum length of the longest leaf; (C) mean relative growth rate of the longest leaf; (D) mean quantum yield. All means are with \pm standard error. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Letters correspond to *post hoc* Tukeys Test differences based on the priming-by-temperature interaction.

After three months of the heatwave there was no significant effect of priming on leaf morphology measures, but there was a significant interaction between priming and temperature on leaf number ($p = 0.02$, Table 2). The number of leaves was significantly higher in the primed seedlings grown under elevated conditions compared to primed seedlings grown under ambient conditions 7 vs 5 leaves, but this effect was not observed in unprimed seedlings, both the ambient and heatwave treatments had on average 6 leaves (Figure 5A). There was an effect of the heatwave temperature on leaf length ($p < 0.05$), leaf necrosis ($p < 0.001$), the relative growth rate of the leaves ($p < 0.001$) and root length ($p < 0.001$, Table 2). Leaf length was significantly lower in the heatwave treatments (75-82 mm) compared to the ambient conditions (82-86 mm, Figure 5B, Table 2). The same result was observed for root length, heatwave: 77 - 79 mm vs ambient: 102 - 112 mm (Figure 7A, Table 2). There was a greater proportion of the leaves with necrosis 60% vs 30% under heatwave conditions (Figure 6, Table 2) and the relative growth rate was significantly lower in the heatwave treatment (Figure 5C, Table 2). There was a significant interaction between the priming and heatwave treatment for photosynthetic yield (p

= 0.006, Figure 5D) where the yield of unprimed seedlings was higher than primed seedlings but only under heatwave, not ambient conditions. There was no effect of any treatment on the number of roots (Figure S5).

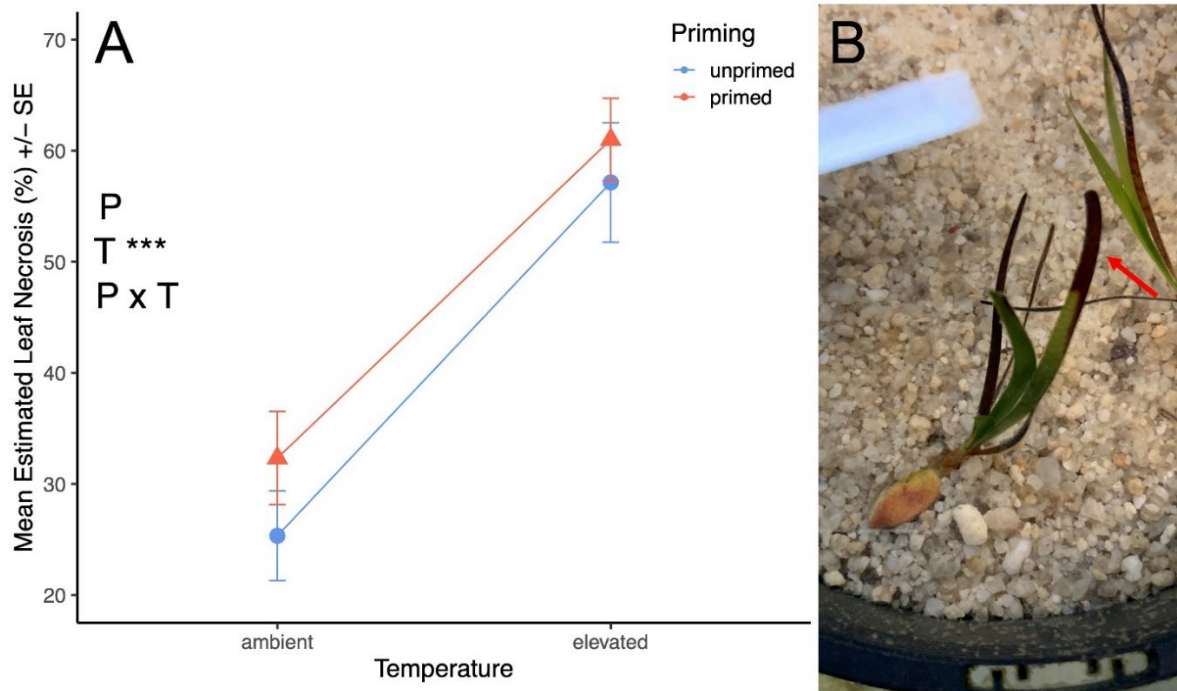


Figure 6: (A) Mean proportion of leaf necrosis (\pm standard error) at the end of the 3-month simulated heatwave scenario, ***, $p < 0.001$; (B) Image of a *P. australis* seedling *in situ* during the 3-month temperature treatment, red arrow points to leaf necrosis on the seedling.

3.2.2 Biomass

There were no significant differences in the total seedling, seed nor leaf biomass at the end of the heatwave treatment, but temperature impacted the root biomass, with lower biomass under heatwave conditions (Figure 7A and 7F, Table 2). There was an interaction between the leaf to root ratio ($p = 0.014$) where there was an increase under heatwave conditions for unprimed seedlings (2.5 vs 1.4), so there was more leaf biomass relative to root biomass, but this was not the case for primed seedlings where there was no difference between ambient or heatwave conditions (~ 2.0) (Figure 7G, Table 2).

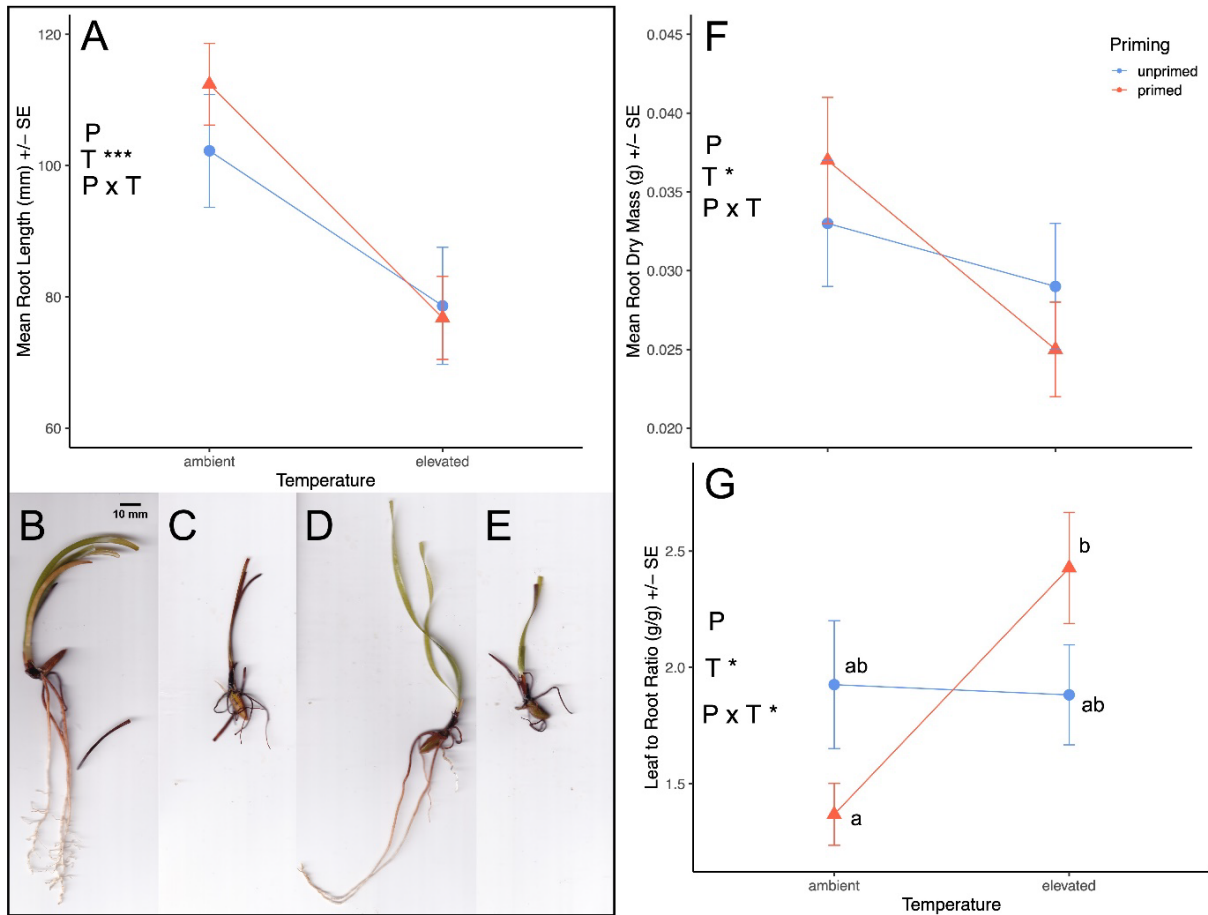


Figure 7: (A) Mean root length (\pm standard error) at the end of the 3-month simulated heatwave. (B-E) Examples of harvested seedlings for Unprimed-Ambient (B), Unprimed-Elevated (C), Primed-Ambient (D), and Primed-Elevated (E) Unprimed-Elevated, (F) Mean root biomass (\pm standard error), and (G) Mean leaf to root dry mass ratio (\pm standard error). * $p < 0.05$, and *** $p < 0.001$. Letters correspond to *post hoc* Tukey's Test differences based on the priming-by-temperature interaction.

Table 2: Shapiro-Wilk test with p -value and two-way ANOVA F and p -values for each trait among treatments after 3 months. Significance is indicated by *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Degrees of freedom (df) = 1 priming, temperature, priming by temperature interaction.

Trait (units)	Trait Abbreviation	Data Type	Shapiro-Wilk	p -value	ANOVA Residual df	Priming (P)		Temperature (T)		PxT Interaction	
						F	p -value	F	p -value	F	p -value
Leaf Number (n)	nLeaf	Rank Transformation	0.903	1.392 E-07	116	0.437	0.510	13.693	0.0003 ***	5.510	0.020 *
Maximum Leaf Length (mm)	Length _{Max}	Normal	0.991	0.546	116	0.748	0.389	4.740	0.031 *	3.677	0.057
Relative Growth Rate of Maximum Leaf Length (mm day ⁻¹)	RGR _{LengthMax}	Normal	0.989	0.416	116	0.412	0.522	22.304	6.190 E-06 ***	2.289	0.133
Photosynthetic Yield of Photosystem II	Fv/Fm	Normal	0.976	0.671	28	2.094	0.159	0.154	0.698	8.769	0.006 ***
Proportion of Necrosis on the Leaves (%)	Brown _{Prop}	Log Transformation	0.965	0.002	116	0.019	0.890	107.725	< 2.00 E-16 ***	0.083	0.773
Number of Roots (n)	nRoot	Log Transformation	0.925	0.0008	55	1.995	0.163	1.831	0.181	0.441	0.509
Maximum Root Length (mm)	Root _{MaxLength}	Normal	0.972	0.152	55	0.291	0.592	17.000	0.000 ***	1.304	0.258
Leaf Biomass (g)	Biomass _{Leaf}	Normal	0.984	0.552	55	0.003	0.957	0.014	0.908	1.301	0.259
Seed Biomass (g)	Biomass _{Seed}	Rank Transformation	0.949	0.010	55	0.336	0.565	1.638	0.206	1.374	0.246
Root Biomass (g)	Biomass _{Root}	Normal	0.969	0.109	55	0.004	0.952	4.186	0.045 *	1.464	0.231
Total Biomass (g)	Biomass _{Total}	Normal	0.988	0.809	55	0.031	0.861	1.939	0.169	0.955	0.332
Leaf to Root Ratio	Leaf:Root	Rank Transformation	0.842	9.605 E-07	60	0.174	0.678	10.819	0.002 **	6.402	0.014 *
Survival (n)	Survival	Log Transformation	0.789	0.002	12	0.675	0.427	10.412	0.007 **	0.675	0.427

3.3 Post Heatwave Survival

Seedling survival declined after the heatwave in all treatments (Figure 8). Higher survival was observed in the ambient temperature treatments at all time points, and this was statistically significant at T24, three months post-heatwave (≥ 87 vs 56 %, $p = 0.008$) and at T36, six months post-heatwave (62 - 69 vs 37 - 44 %, $p = 0.026$).

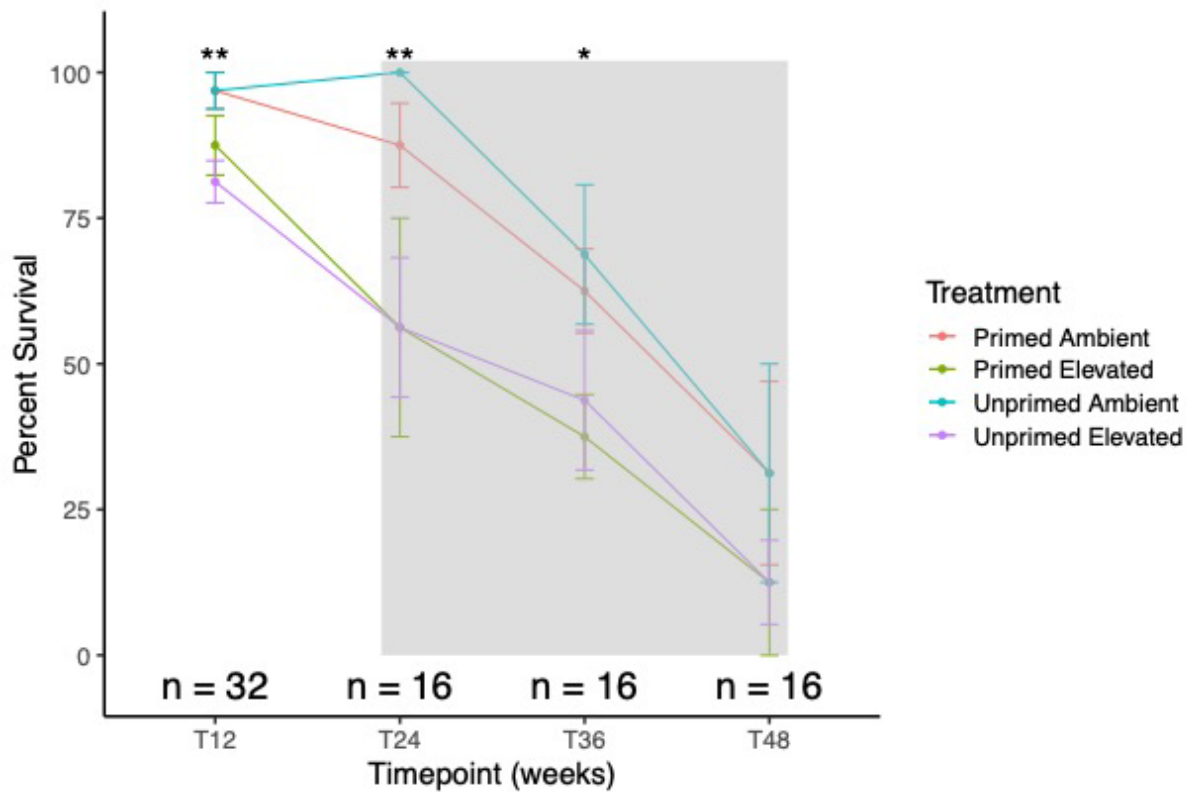


Figure 8: Mean percent survival of *P. australis* seedlings (\pm standard error) every three months from the conclusion of the 3-month heatwave experiment up to 1-year following fruit dehiscence. **, $p < 0.01$, *, $p < 0.05$.

4 Discussion

Conserving and restoring natural ecosystems is among one of the most effective strategies to mitigate the effects of climate change (Cook-Patton et al., 2021) but as rapid warming is impacting biodiversity (IPBES, 2019) direct interventions to future-proof populations to warming is of great interest (Coleman et al., 2020, Gaitan-Espitia and Hobday, 2021). Priming is one such intervention strategy where exposure of individuals to a particular environmental stress such as higher temperatures can reduce sensitivity to subsequent exposures improving resilience to future conditions. We trialled this resilience building strategy with a wide-spread and ecologically significant seagrass species, *P. australis* with seeds that are used in existing restoration programs. We found no significant positive effect of thermal priming to 26 °C on growth, biomass and survival of *P. australis* seedlings following re-exposure to a simulated three-month Category IV MHW (28 °C) and also after an additional nine months when the seedlings were one year of age. Where there was an effect of priming, negative effects under MHW conditions were observed, such as reduced photosynthetic health and a trade-off between investment in above and below-ground biomass. There is no support to use this approach as a resilience building strategy in seed-based restoration programs for *P. australis* based on the conditions we imposed, as primed seedlings did not perform better than unprimed seedlings under MHW conditions.

Priming has been tested as a potential method to build resilience in temperate seagrasses to extreme thermal events for adult plants and seedlings (DuBois et al., 2020; Nguyen et al., 2020; Pazzaglia et al., 2022, Jueterbock et al., 2021), but this is the first study where priming has occurred on the seed after it was released from the fruit and plants have been monitored for a long duration after the MHW was stopped. This longer duration is valuable to understand as other studies that imposed warming treatments only found negative effects manifesting during the recovery phase (Beca-Carretero et al., 2021). Enhanced heat resistance measured through photophysiology, growth and investment in shoots and leaves, as well as activation of genes have all been identified through priming in *Zostera* adult plants and *Posidonia* adults and beach-cast seedlings (Jueterbock et al., 2021). This enhancement is generally due to a less severe negative effect on the trait compared to an unprimed plant (*Zostera marina*, DuBois et al., 2020; *P. australis* and *Zostera muelleri*, Nguyen et al., 2020), or in the case of Pazzaglia et al. (2022), there was a positive impact of priming where priming resulted in elevated growth rates when compared to the controls or unprimed *P. oceanica* plants. This did not translate into significantly different leaf surface area increases suggesting that seedlings exposed to the MHW were still under thermal stress. Interestingly in Nguyen et al. (2020) only physiological, not growth responses were enhanced for *P. australis* adult plants with priming, but both were enhanced in *Z. muelleri*, highlighting species differences that could be linked to life-history strategy. Although Pazzaglia et al. (2022) demonstrated growth responses in *Posidonia* seedlings highlighting differences due to life-stage. This is not surprising as there are ontogeny-specific differences in thermal tolerance within *Posidonia* (Rinaldi et al., 2023).

The absence of a priming effect in our study could be an artefact of the young age of the plants. They were primed within days of being released from their buoyant seed coat, so would be totally reliant on the seed endosperm for growth, unlike the 3-month old *Posidonia* seedlings assessed by Pazzaglia et al. (2022) which would have less reliance on the endosperm and this would not be the case when using mature ramets or fragments (DuBois et al., 2020; Nguyen et al., 2020). Another aspect to consider is that the experiments to date have all used different combinations of priming and heatwave (sometimes called 'triggering') treatments, in terms of both the duration and magnitude of the treatments, the period after the heatwave that the plants were monitored for, as well as the species and life-cycle stage. Our study is the longest MHW duration and the longest monitored to date, post-heatwave showing that over this time priming effects did not manifest. Different experimental conditions related to the temperature and duration for priming, the optimum recovery (or re-acclimation) period, and the temperature and duration for re-exposure could be explored to identify if a priming benefit exists for *P. australis* seedlings under different conditions.

Despite not identifying priming as a possible resilience building strategy, this study does provide insights into how *P. australis* seedlings will respond to MHWs that can be used for future predictions. The simulated MHW of 28 °C for three months, analogous to a Category IV MHW (Hobday et al., 2018) in a region where the ambient summer temperatures are 22 °C resulted in higher seedling mortality, reduced growth, reduced leaf length with more necrosis and reduced root length and biomass. This is analogous to other warming studies that have demonstrated negative impacts with warming (Webster et al., in review, Strydom et al., 2020, Hammer et al., 2018). The growth rates and seedling biomass produced were similar to the two studies we are aware of that have measured seedlings in early stages, all from mesocosm not field conditions (Glasby et al., 2015, Statton et al., 2014), giving confidence that the responses were measured were realistic. For young seedlings, establishment via elongation of the primary root and subsequent lateral roots is critically important for anchoring and lack of successful anchoring is a key reason for high mortality rates in newly establishing *P. australis* seedlings within the first year (Statton et al., 2017; Kendrick et al., 2023). Warming during the young seedling stage with the associated reduction in roots will likely have a very negative effect on the seedling survival, increasing this bottleneck.

Posidonia australis seeds are released in very early summer when MHWs are common (Oliver et al., 2018), and the frequency of MHWs is predicted to increase (Cai et al., 2014), therefore, long-term implications may exist for natural seagrass recruitment processes and the maintenance of genetic diversity (Kendrick et al., 2023). For example, the survival of seedlings after the heatwave ceased, tracked up to 1 year of age was much higher in our study, 12 - 31 % than the estimated 1 % rate survival from field observations (Statton et al., 2017), and it is this 1 % that could potentially be reduced due to the effects of MHWs. Reduced investment in below-ground biomass with MHWs has also been observed in adult plants (DuBois et al., 2020). But for this life-history stage there is a clear benefit in reducing respiratory load under warmer conditions particularly linked to minimising the risk of sulfide intrusion and shoot mortality (García et al., 2012). Therefore, when making predictions about the impacts of MHWs on the resilience of seagrass populations the implications to different life-stages should be considered.

Several complex physiological processes are associated with climate, or environmental adaptation in plants (Allen et al., 2010). Trade-offs between growth and productivity under both biotic and abiotic stressors are common (Darychuk et al., 2012; MacTavish and Anderson, 2020; Meira-Neto et al., 2019). This was demonstrated with both the number of leaves and leaf length and the allocation of resources to above and below-ground components. Primed seedlings at ambient temperatures had the fewest number of leaves but exhibited the greatest maximum length while primed seedlings at elevated temperatures had the highest number of leaves but were also the shortest, consistent with a known temperature trade-off for growth in terrestrial plants (Sterck et al., 2006; Whitman and Aarssen, 2010). Production of more leaves by primed plants under MHW conditions may be a stress response where the seed's starch and nutrient resources are preferentially allocated into new growth, an observation that has been observed under hyposaline stress in *Halophila ovalis* and *Z. muelleri* (Collier et al., 2014). This response is to the detriment of below-ground material, especially roots, and the implications of this are explained above.

While this experiment was applied as an extreme scenario, it is one that has occurred in Western Australia, resulting in population declines or changes in species assemblage (Nowicki et al., 2017), and could occur with increased frequency in the future. Under extreme future climate scenarios e.g. Category IV MHW (Hobday et al., 2018; RCP 8.5 (IPCC, 2018), increased frequency, duration, and intensity of MHWs may impact future seedling establishment success, particularly when current natural seedling survival rates are already low (Statton et al., 2017). It is likely that a combination of methods (e.g. translocations, genetic rescue, and priming of mature plants) and life history stages (seeds, adult plants) may need to be employed to maximise restoration efforts. Further empirical testing in a field environment could provide greater insights in Australian *Posidonia* recruitment and thermal tolerance.

Conclusions and recommendations

We highlight the importance of understanding the growth and responses of the most vulnerable life history stage, seed(lings) to a severe marine heatwave. Thermal priming of *P. australis* seed(lings) before planting and then grown at ambient or elevated temperatures simulating a MHW did not enhance survival or growth rate during the first year, despite the method showing promise to build thermal tolerance in mature *P. australis* plants. Furthermore, we find that growth and survival were negatively impacted when subjected to a MHW with implications for seedling establishment in natural settings. We recommend additional thermal priming experiments be conducted to fully understand the dynamics and responses under a range of initial (priming) and re-exposure (triggering) durations and intensity. Other experiments using seedlings from environments where plants may be warm-adapted as identified through our population genomics study for sister taxon, *P. sinuosa* (Whale et al., 2024 in review) could also be employed. Seed priming should not be applied to *P. australis* seed(lings) to build thermal resilience using the conditions trialled in this study.

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7 Appendices

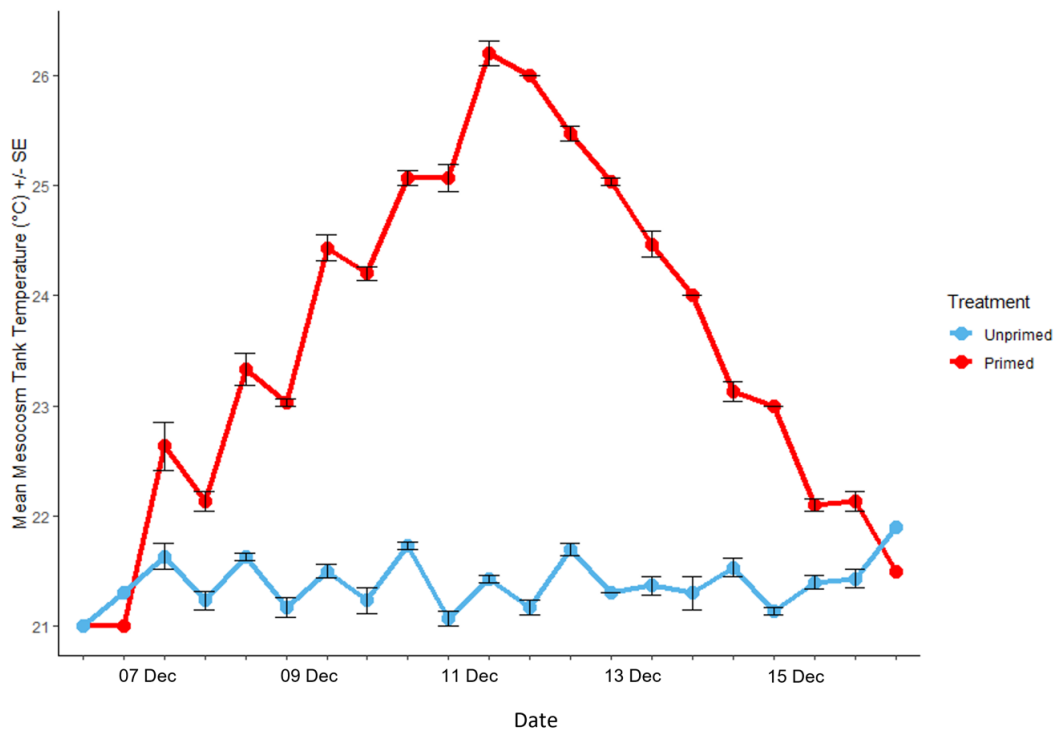


Figure S1: Changes in water temperature (\pm standard error) between the two priming regimes during the 10-day priming event.

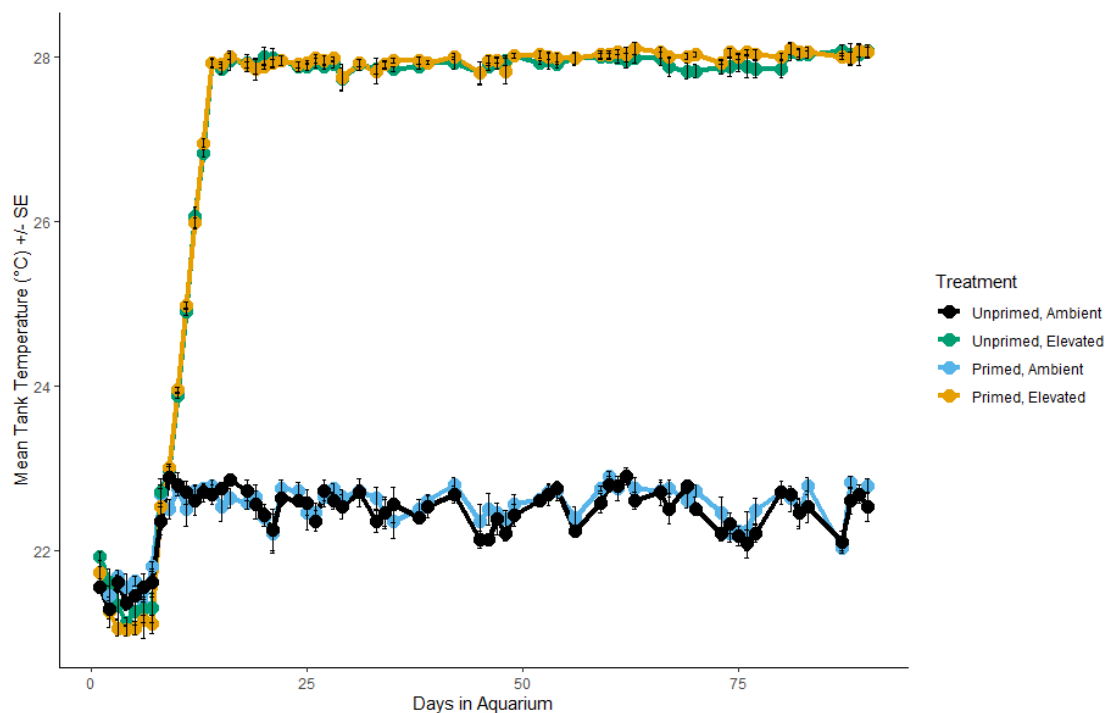


Figure S2: Daily tank water temperature (\pm standard error) for all treatments during the 3-month simulated heatwave phase of the experiment.

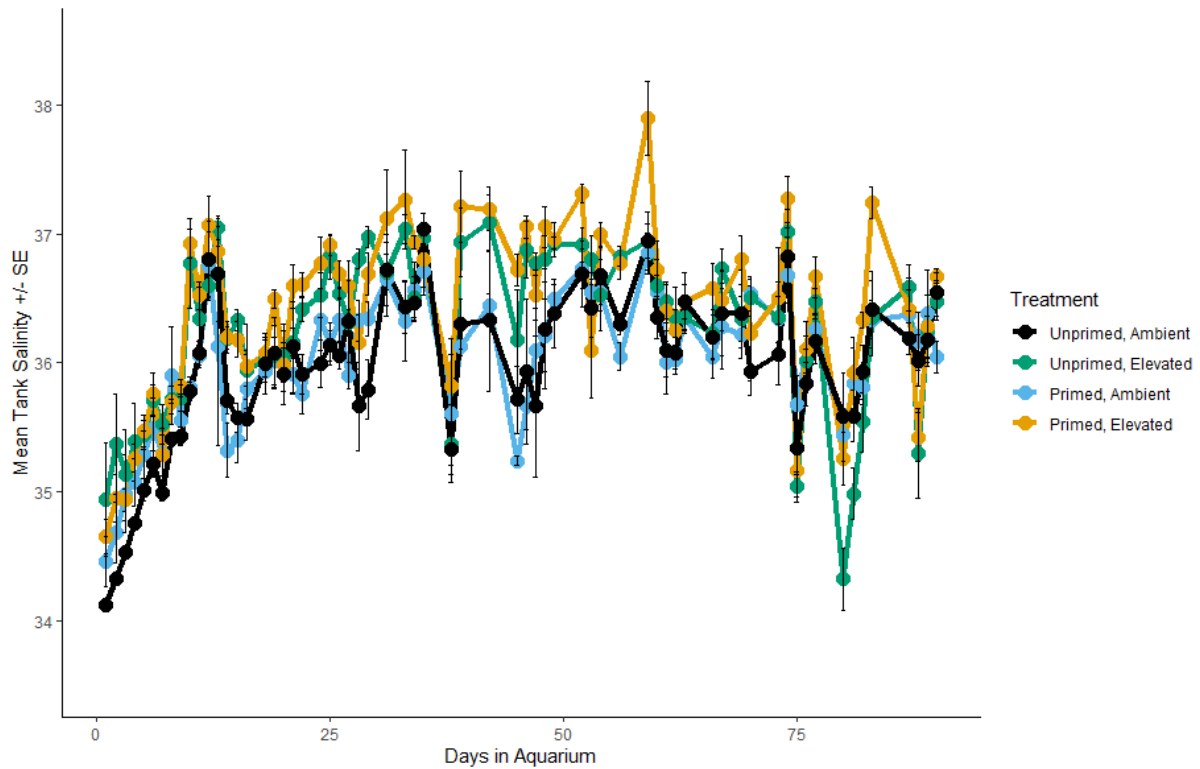


Figure S3: Daily tank water salinity (ppt) with (\pm standard error) for all treatments during the 3-month simulated heatwave phase of the experiment.

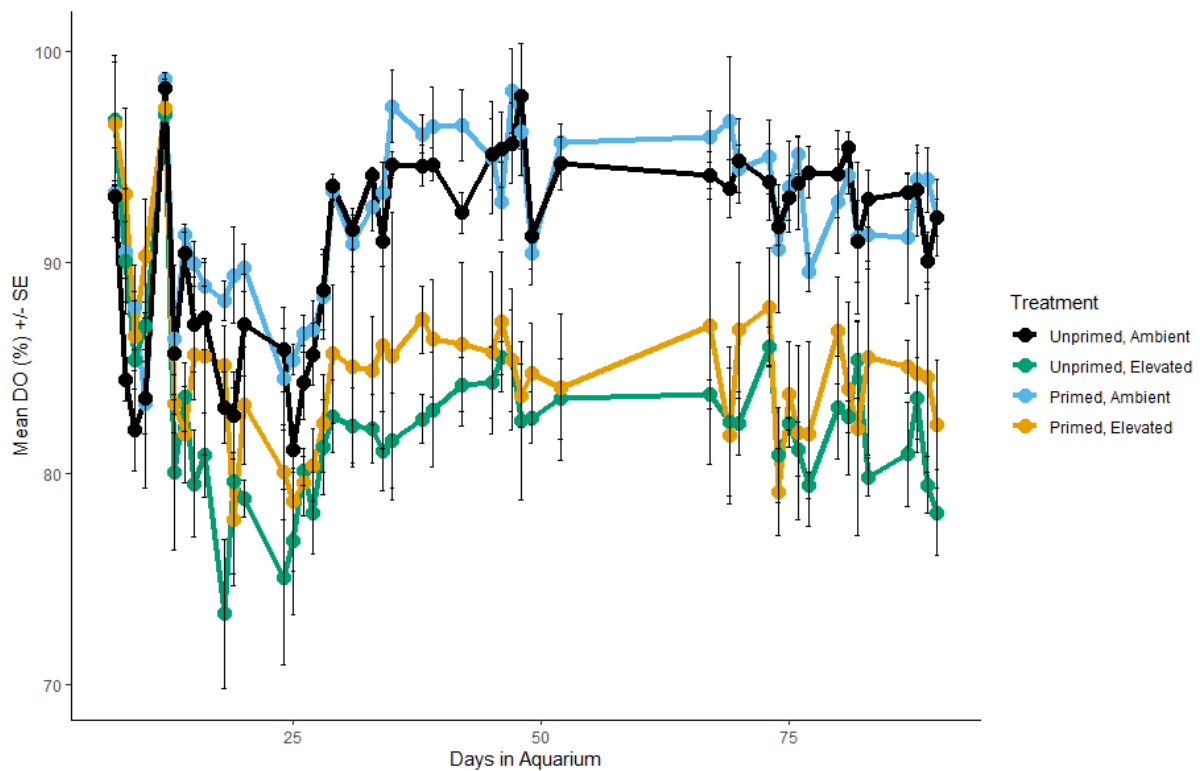


Figure S4: Daily tank water dissolved oxygen (\pm standard error) for all treatments during the 3-month simulated heatwave phase of the experiment.

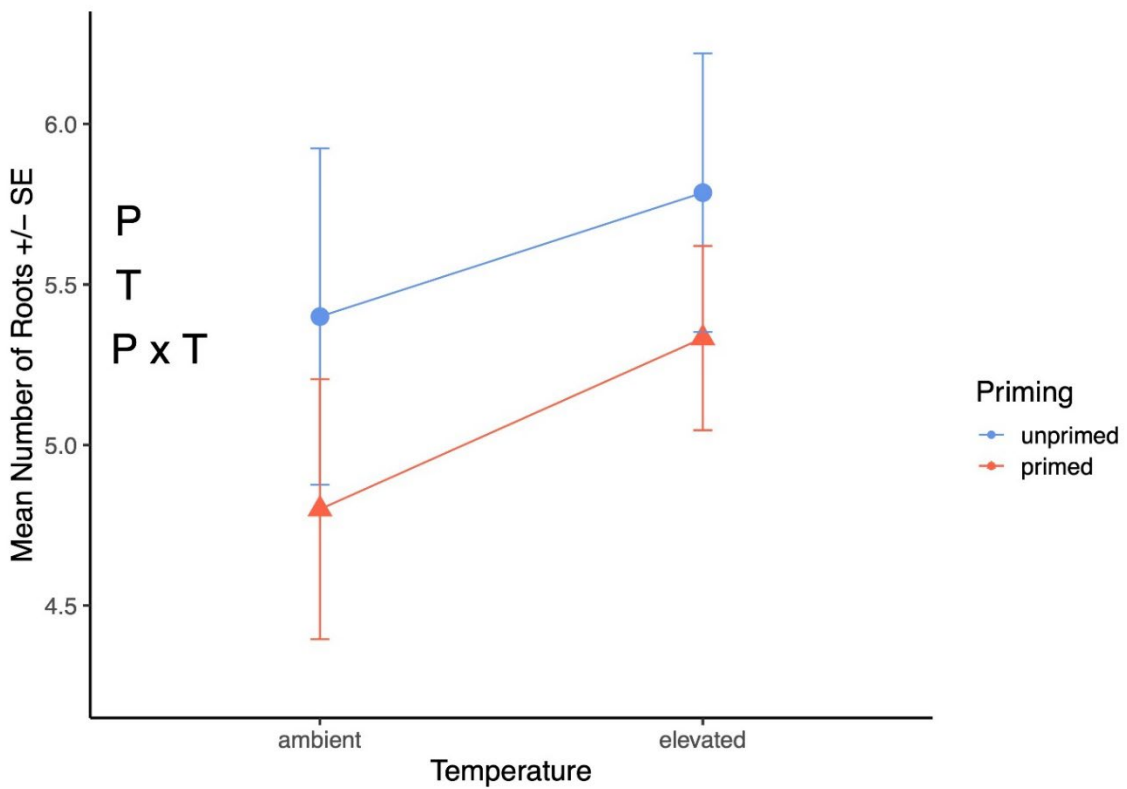


Figure S5: Mean number of roots (\pm standard error) among treatments at the end of the 3-month simulated heatwave.

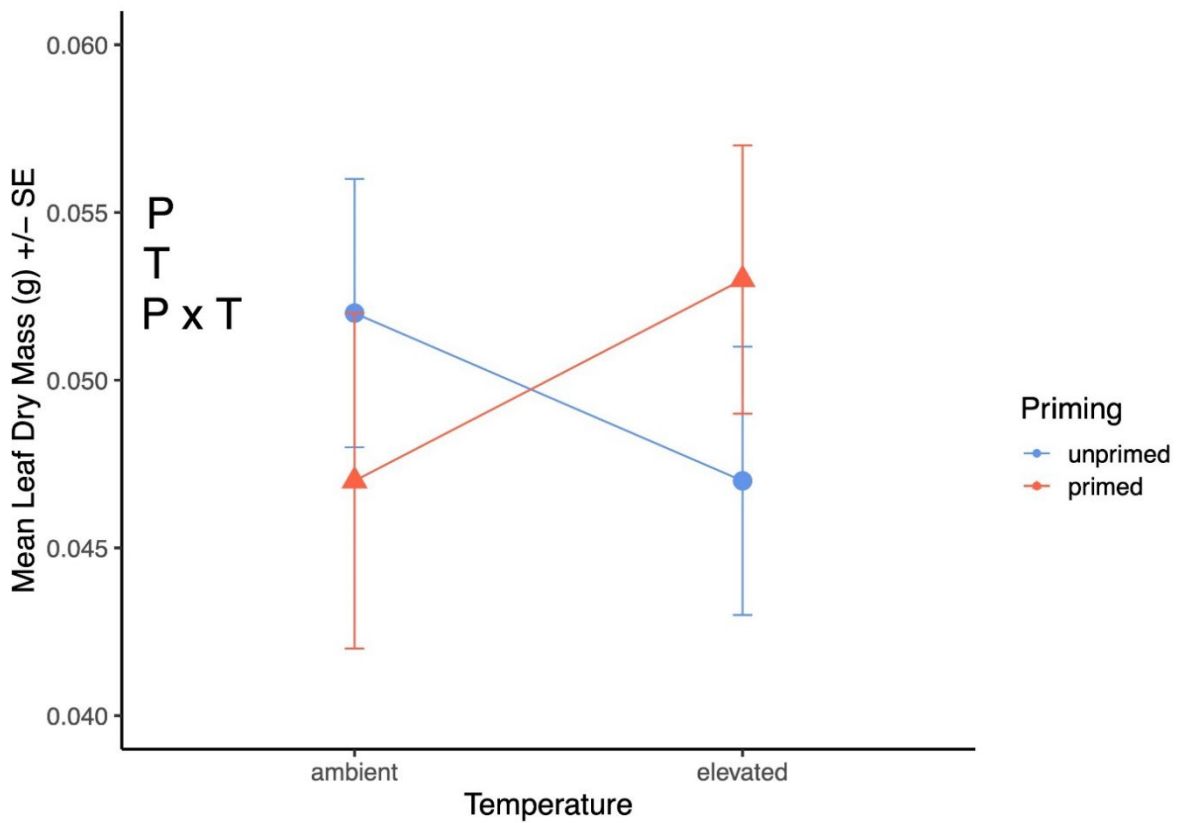


Figure S6: Mean oven-dry leaf biomass (\pm standard error) among treatments at the end of the 3-month simulated heatwave.

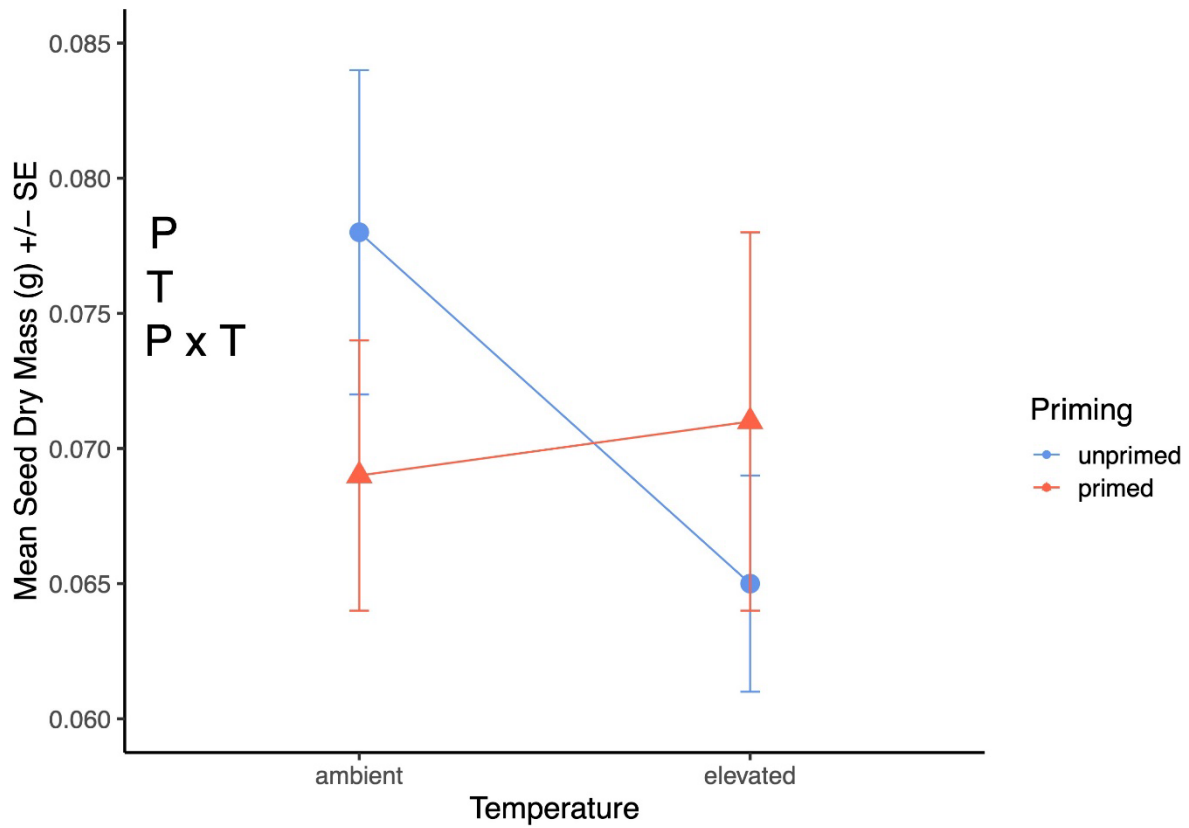


Figure S7: Mean oven-dry seed biomass (\pm standard error) among treatments at the end of the 3-month simulated heatwave.

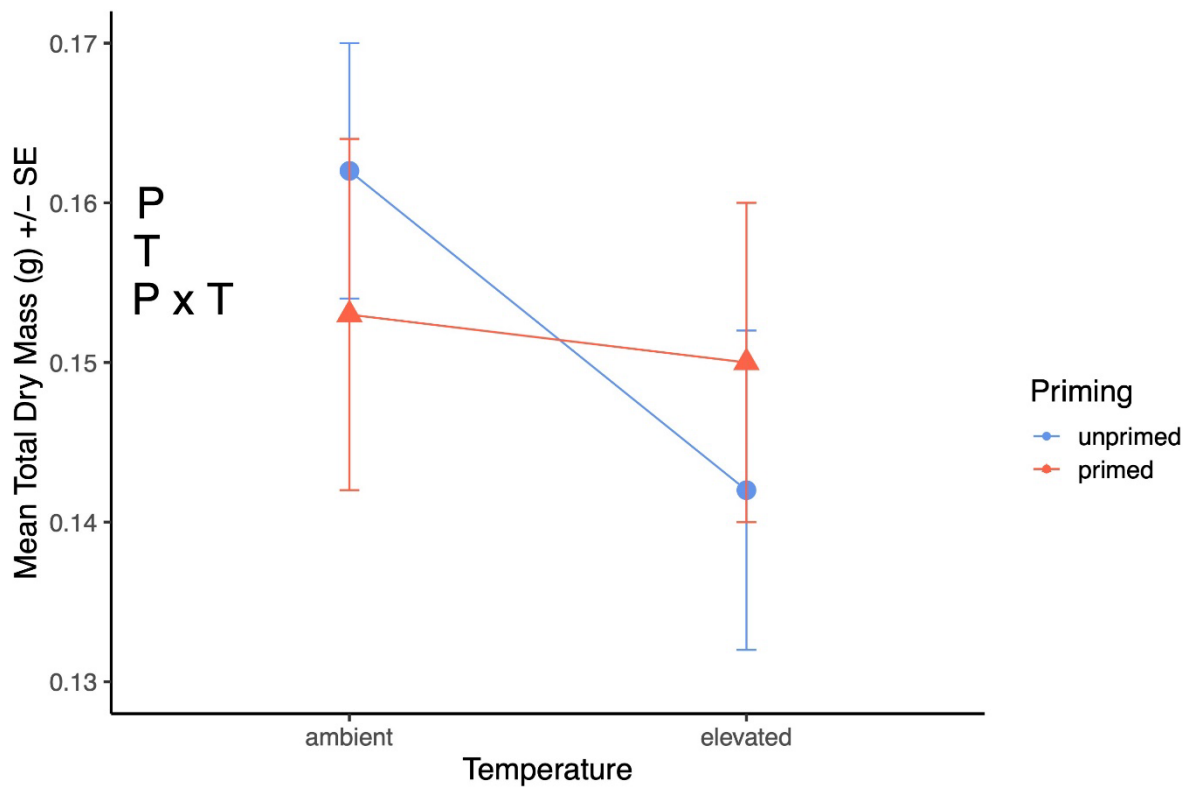


Figure S8: Mean oven-dry total biomass (\pm standard error) among treatments at the end of the 3-month simulated heatwave.

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