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Chronically elevated sea surface temperatures revealed high susceptibility of the eelgrass *Zostera marina* to winter and spring warming

Yvonne Sawall,[1,2]* **Maysa Ito**,[1,3] **Christian Pansch**[1,4]

[1]Department of Marine Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[2]Bermuda Institute of Ocean Sciences (BIOS), St. George's
[3]Ifremer, Channel and North Sea Fisheries Research Unit, Boulogne-sur-mer, France
[4]Environmental and Marine Biology, Åbo Akademi University, Åbo, Finland

Abstract

While it is well known that severe marine summer heatwaves can cause acute and dramatic die-offs of seagrass meadows, the effect of trans-seasonal warming and winter/spring heatwaves are yet poorly understood. This study simulated a 9-months warming scenario on the common seagrass *Zostera marina* from winter into summer, using outdoor mesocosms, which provided near-natural conditions. The relevance of the natural temperature pattern, as well as the 3.6°C warming, and their implications were further discussed in the context of a 22-yr temperature time series of the study region. Survival of plants was high in winter independent of temperature. In spring, however, heat-treated *Z. marina* flowered 1.5 months earlier and experienced high mortalities. Thereafter, plant survival, growth, and pigmentation were largely comparable between temperature regimes. Yet, a comparatively high mortality occurred in ambient plants, after an abnormally warm June. Final biomass was reduced by ~ 50% in heat-treated plants. These results imply that warm winter-to-spring conditions can have severe effects on vital seagrass traits. Warming accelerates consumption of energy reserves triggering advanced flowering, similar to many terrestrial plants. Although, surviving heat-treated plants were not able to re-stock energy reserves throughout the high-light summer as inferred from low plant biomass, these seemed rather resistant to summer heatwave events.

Global warming is a major concern for many shallow water coastal ecosystems (Gattuso 2018) and poleward range shifts have been documented for marine ecosystems worldwide (Pinsky et al. 2013), with far-reaching consequences for biodiversity (Burrows et al. 2019). Global warming affects trends and regimes of sea surface temperature (SST). These include an increase of mean annual SST, and an increase in the severity and frequency of marine heatwaves and increasing magnitude of temperature fluctuations (due to more extreme weather events—calm periods and storms). Globally, annual SST has increased by > 0.1°C per decade since 1971, while in the Baltic Sea, SST of the coolest month has even increased by 0.35°C per decade since 1950 (IPCC 2014) representing a warming hotspot (Reusch et al. 2018). The occurrence of marine heatwaves is characterized by temperatures that substantially exceed average mean SST over a prolonged period of time. Hobday et al. (2016) define marine heatwaves as a period lasting "for five or more days, with temperatures warmer than the 90th percentile based on a 30-year historical baseline period," allowing the detection of heatwave events in nature for monitoring their impacts on community structure and functioning (Jentsch et al. 2007; Garrabou et al. 2009; Wernberg et al. 2013).

Seagrass meadows have a global distribution framing numerous shorelines and provide a number of ecological (e.g., habitat, nutrient cycling, food, sediment stabilization; Smith 1981; Krause-Jensen and Duarte 2016) and economic services (e.g., fisheries, coastal protection, blue carbon; Holdt and Kraan 2011; Costanza et al. 2014; Röhr et al. 2016). The seagrass *Zostera marina* is the most common seagrass species

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*Correspondence: yvonne.sawall@bios.edu

Additional Supporting Information may be found in the online version of this article.

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along temperate latitudes of the northern hemisphere (Den Hartog, 1970) spanning a wide range of environmental conditions and featuring region-specific adaptations (e.g., different temperature optima depending on local temperature regimes; Lee et al. 2007; Beca-Carretero et al. 2018).

The most studied global warming phenomena in mid-latitude seagrass meadows are marine summer heatwaves (Bergmann et al. 2010; Reynolds et al. 2016; Ruiz et al. 2018). Field observations following these extreme events of up to 5°C above mean summer SST for > 3 weeks have detected severe seagrass die-off in Western Australia (Arias-Ortiz et al. 2018; Strydom et al. 2020), the Mediterranean (Marbà and Duarte 2010), and the Baltic Sea (Reusch et al. 2005). Experimental studies of near-natural interim heatwaves (e.g., 5°C SST anomaly over 5–9 d), as they occur frequently in shallow coastal areas, however, have shown little immediate effects on the performance of *Z. marina* in the Baltic Sea (Saha et al. 2020). Yet, longer-term (e.g., 4–6°C SST anomaly over 3–6 weeks) manipulation experiments reported a number of stress responses inducing upregulation of heat-stress related genes (e.g., heat shock proteins, superoxide dismutase; Bergmann et al. 2010; Franssen et al. 2011; Franssen et al. 2014), and reduction in photosynthetic performance and growth (Winters et al. 2011; Repolho et al. 2017; Ruiz et al. 2018). Seagrass seedlings may in particular be susceptible to heat stress due to reduced growth and high mortality during heatwave events (Olsen et al. 2012; Guerrero-Meseguer et al. 2017). In adult plants, signs of heat stress such as reduction of growth and biomass are not always visible during the heat-stress exposure, but become evident only after several weeks (Bergmann et al. 2010; Reynolds et al. 2016). The occurrence of such lag effects and the ability to recover from heat-stress events was shown to differ between species and population, largely depending on thermal history (Franssen et al. 2011; Winters et al. 2011).

In contrast to summer heatwaves, the (potential) impacts of winter and/or spring warming on seagrass ecosystems are mostly unknown (but *see* Traboni et al. 2018). Particularly, the effect of chronically increased SST across seasons has not yet been studied in seagrass systems. In terrestrial grasslands, winter warming caused clearly stronger responses than warming applied in summer, entailing a higher primary production on the one hand but a higher sensitivity to frost, and a reduction in diversity on the other hand (Kreyling et al. 2019). Furthermore, a number of studies on terrestrial plants have shown that warm winter and/or spring seasons can cause advanced flowering, including a number of grass species (meta-analysis by Cook et al. 2012), which may or may not have negative ecosystem consequences (Hegland et al. 2009; Rafferty and Ives 2011). There is evidence that temperature-driven advanced flowering may also be true in seagrass meadows, based on the finding that flowering of *Z. marina* is earlier in warmer low-latitude seagrass meadows than in cooler high latitudes (Blok et al. 2018). The same study, however, also showed that *Z. marina* features different temperature sensitivities at different latitudes likely due to local adaptation (Blok et al. 2018).

Unlike for light (Fitzpatrick and Kirkman 1995; Ruiz and Romero 2001) and nutrients (Fourqurean et al. 1995; Worm et al. 2000), long-term SST manipulation experiments are rare as these cannot be conducted in situ. In situ transplantation experiments between sites of differing SST regimes are possible (Macreadie and Hardy 2018); however, replication remains a challenge and this approach usually also entails changes in other environmental conditions, which may produce confounding results. Therefore, near-natural long(er)-term ex situ manipulation experiments are required to investigate the effect of chronically increased SST, which are now possible using advanced mesocosm technology.

The objective of this study was to investigate the effects of chronically increased SST on the foundation seagrass species *Z. marina* over a period of 9 months spanning across three seasons (winter–autumn–summer, December 2015 to August 2016; northern hemisphere). In particular, the effect of SST rise in winter has rarely been studied experimentally in marine plants but may be equally important as summer marine heatwaves to understand the effects of global warming. We hypothesized that abnormally warm winters or springs will likely not result in a direct heat-stress response of seagrass plants, but rather in an imbalance of energy reserves caused by increased metabolic rates and light limitation. In particular in winter, respiration rates may be sensitive to temperature changes due to seasonal acclimation of metabolic processes (Staehr and Borum 2011). A12-tank outdoor mesocosm facility (Wahl et al. 2015) was used to simulate near-natural habitat conditions and to implement a warming scenario of 3.6°C above the ambient temperature measured in situ at any given time. Temperature treatments were placed in context to a prevailing and local 22-yr average SST time series, which allowed identifying and characterizing SST anomalies.

Material and methods

Species and study site

The eelgrass *Z. marina* occurs in the Baltic Sea from Denmark to southern Finland (Boström et al. 2014) along a strong environmental gradient (i.e., salinity and sediment organic matter) that determines primary production and epiphytic load (Holmer et al. 2009). Within the southwestern Baltic Sea, *Z. marina* occurs at depths ranging between 0.5 and 8 m with highest densities between 3 and 5 m (Schubert et al. 2015). Over the last decades, an increase in nutrient loads entailed a decrease of light penetration through the water column, limiting the depth distribution of seagrass (Krause-Jensen et al. 2011). Moreover, the nutrients deposited in the sediment are related to the increase of sulphide, which intrudes the rhizomes whenever *Z. marina* is under any other stress. This process disrupts the internal pO_2, resulting in high mortality of shoots (Pedersen et al. 2004). In 2003, an extreme summer heatwave led to the reduction of biomass in a southwestern Baltic Sea population of *Z. marina*, from which only high genotypic variability in the population allowed partial recovery (Reusch et al., 2005).

For this experiment, the eelgrass *Z. marina* was collected on 24 November 2015 in the Kiel Fjord (Falckenstein: 54°24′24.7″N, 10°11′38.7″E), southwestern Baltic Sea, along a 100 m transect at 2–3 m depth within a perennial eelgrass meadow. Whole plants (1–3 shoots per plant) were kept in a cooler filled with fjord water and immediately transported to the experimental facilities. Individual shoots (each from a different plant) of similar size (shoot length: 6–10 cm; rhizome length: 3–5 cm) were used for the experiment. For this, sediment, previously collected and stored in aerated barrels, was placed into 28 × 20 cm boxes of 5 liters, into which six shoots of *Z. marina* were planted. During an acclimation period of 7 d, the shoots were maintained under ambient fjord temperature.

The experimental community was designed based on the composition of a typical shallow water coastal ecosystem of the Baltic Sea that is comprised of seagrass meadows and *Fucus* belts. The species introduced in the tanks in addition to the habitat forming macrophytes *Z. marina* and *Fucus vesiculosus* were the gastropod *Littorina littorea*, the isopod *Idotea balthica*, amphipods of the genus *Gammarus* spp., the decapod *Palaemon* sp., and bivalves of the species *Mytilus edulis*. Other organisms that accessed the tanks due to the inflow of non-filtered fjord water (*see* below) were filamentous algae, polychaetes (e.g., *Amphitrite* sp., *Eteone longa*, *Harmothoe imbricata*, *Marenzelleria* sp., *Polydora cornuta*, *Hediste diversicolor*, *Scoloplos* sp.), other bivalves (e.g., *Limecola balthica*, *Mya* sp., *Mysella* sp.), gastropods (e.g., *Hydrobia ulvae*, *Retusa* sp.), and round goby (*Neogobius melanostomus*). For comparisons on the species assemblage and approach, *see* also Pansch et al. (2018) and Saha et al. (2020).

Experimental design

The experiment was carried out from 25 November 2015 to 26 August 2016 (across three seasons: winter, spring, and summer) in the Kiel Outdoor Benthocosms (KOBs; Wahl et al. 2015), located at the inner Kiel Fjord (54°19′47.74″N, 10°8′58.14″E). The KOBs are composed of 12 experimental units characterized by thermal insulation, a capacity of 1500 liters and wave generators that, combined with pumps, induce water movement, in order to avoid stratification. The system provides near-natural conditions as the units are supplied with natural light and a constant flow-through of non-filtered water from the fjord, at rates of about 1800 liters per tank per day. The flow-through and the metabolic activity of the community enables natural fluctuations in abiotic parameters (pH, nutrients, salinity, oxygen and particulate, and dissolved organic matter) and the access of organisms that settle and populate the units. Each experimental unit is equipped with a Profilux controller system (GHL Advanced Technology) synchronized with heaters and chillers that maintain target temperatures.

The experiment included two levels of temperature (*Ambient* and *Heat*, i.e., 0 and 3.6°C above naturally fluctuating fjord temperatures, respectively), which was replicated in six independent tanks for each treatment. The target temperature of the *Heat* treatment was approached by daily temperature increments of 0.3°C over 12 consecutive days (3–15 December 2015), after the 7-days acclimation period. Seasonal, diurnal, and stochastic fluctuations were maintained in both treatments throughout the experimental duration. The *Heat* treatment chosen represents a future warming scenario for the Baltic Sea to be expected within the next several decades (Gräwe et al. 2013). The Baltic Sea increased 0.35°C per decade from 1950 to 2009 in the coolest months (Hoegh-Guldberg et al., 2014), while the average temperature of the Baltic Sea increased 1.35°C from 1982 to 2006 (Hoegh-Guldberg et al., 2014).

Monitoring of abiotic parameters

In each tank, temperature and dissolved oxygen were logged every 10 and every minute, respectively (Hach-Lange, 4H.Jena Engineering GmbH). Salinity was measured manually 3 h after sunrise using a WTW multimeter (Multi WTW Cond 3110 1 Tetra Con 325). Water samples (20 mL) for inorganic nutrient analyses (PO_4, NH_4, NO_2, NO_3) were collected monthly (December to April) or bimonthly (April to August). Nutrient samples were filtered through a 0.45 μm cellulose-acetate membrane filter (Sartorius) and the filtrate was stored in polyethylene vials at −20°C until further analysis. Nutrient concentrations were determined with an autoanalyzer (Seal Analytical) following standard protocols (Lewandowska et al. 2014).

Measurements of response traits

Number of original shoots, new shoots, and sexually reproductive shoots were recorded on a bimonthly basis between 26 January and 12 August, but less frequently in February (once) and March (none). Original shoots growing new shoots were identified and counted only at first appearance. Likewise, reproductive shoots were identified based on the occurrence of seed-containing spathes and counted only at first appearance. Percentages were calculated based on the amount of originally introduced shoots (24 per experimental unit = 100%). The number of new shoots was standardized to month to account for the longer sampling intervals in December/January and February/March.

Growth rate, leaf production, lengths of the 3rd leaf, and number of green leaves per shoot were determined once per month starting 16 March. For growth measurements, a small plastic ring open on one side, was carefully inserted through each leaf 3 cm above the base (one ring per leaf), which served as a growth mark (Short et al. 2001; Saha et al. 2020). Extensions of leaves (growth rates) were measured 2 weeks later. The same reference points were used to calculate the duration of growing a new leaf (P_L), and to determine the total length of the 3rd leaf. The 3rd leaf is usually the longest leaf of a shoot and has normally reached its final length. The number of green leaves per shoot was counted when growth measurements were taken.

Pigment concentration was measured on leaf parts sampled once per month (except for March). Chlorophyll *a* (Chl *a*) was analyzed as a measure for photosynthetic pigments and β-carotene was assessed as an approximation for photoprotection (antioxidant and precursor of photoprotective xanthophyll pigments; Silva et al. 2013; Galasso et al. 2017). For this, a 5-cm-long piece was cut from the 2nd leaf of one randomly chosen shoot per box (four shoots per experimental unit), instantly frozen in liquid nitrogen and stored at −80°C until further processing. For pigment analysis, each leaf was freeze-dried before the dry weight was determined, then placed in 0.5 mL 2-propanol, homogenized with a Ultraturrax (20 s), and left at 7°C for 18 h, for pigment extraction. After extraction, samples were centrifuged (15 min, 14,000 rpm, 4°C) and the pigment containing supernatant was analyzed with a Varian HPLC 940-LC. Pigments were separated by a normalphase-column (CC250/4.6 Nucleodur, 100-5; Macherey & Nagel) with a gradient of n-heptane/ethylacetate/n-heptane as eluents. The pigments were identified and quantified with a photodiode array detector. Standards were prepared from HPLC-grade Chl *a* (96145, Sigma-Aldrich) and β-carotene (0303S, Extrasynthese). Concentrations were standardized to g dry weight of *Z. marina* leaf and Chl *a*/β-carotene ratios were calculated.

Final biomass was determined at the end of the experiment after shoot collection on 26 August. All shoots were carefully removed from the sediment and rinsed in seawater to remove any remaining sand particles. Epibionts were rare, but carefully scraped off the leaves if present. Shoots were split into above- (leaves) and below- (roots and rhizomes) ground biomass and frozen at −40°C until further processing. Biomasses of above- and below-ground biomass were assessed separately to the nearest mg (Sartorius; precision < 0.1 mg) after drying at 80°C for 24 h.

Data analysis

The software OriginPro 2018 was used for graphing. To test for significant differences of response parameters between *Ambient* and *Heat* generalized linear mixed effect models (GLMM) were applied using the lme4 package in the software R (Bates et al., 2015). For this, the fixed effect was considered to be the interaction between "treatment" (*Ambient* vs. *Heat*) and "time" (date within experimental period), since it was expected that the treatment effect varies with time of the year. Because the response variables did not follow a linear regression with time, the factor "time" was included as a polynomial term (2nd or 3rd order), as this term provided the best fit based on the AIC values. GLMM for the response variables biomass below and above ground did not include the factor "time," since it was measured at the end of the experiment only. The random factors included "tank" (experimental unit) and nested within each tank the factor "box" (planter box within each experimental unit). Finally, to calculate the probability of a significant effect of the treatment, a "null model" was run for each response parameters (i.e., model that excluded "treatment" as a fixed factor). Then, the "treatment model" and the "null model" of each response parameter were compared with each other using a Chi-square test.

Results

Environmental parameters

Ambient benthocosms closely followed the temperature regime of the Kiel Fjord over the entire experimental period of 9 months (Fig. 1). After the initial ramp up in delta temperature, *Heat* benthocosms were consistently 3.6°C higher than *Ambient* (Fig. 1). The experiment started with a temperature of ~ 10°C in the Kiel Fjord and the experimental units and reached the lowest temperatures in the second half of January 2016, where *Ambient* temperature remained below 4°C over a period of 10 d with minimum values of 1.3°C. Temperatures in *Ambient* reached highest values in June 2016 with 18–20°C and short-term daytime temperatures of up to 22.2°C. Temperatures in *Heat* reached minimum and maximum values of 5.1°C and 26.1°C, respectively. Diurnal fluctuations were strongest with up to 2.5°C during rather steep increases in temperature (coinciding with calm and sunny weather periods) beginning of May, beginning of June and end of July 2016 (Fig. 1). A partial failure of the temperature controllers 5–8 February 2016 led to short-term increase in temperature in some benthocosms by ~ 2.5°C and ~ 1.5°C in *Ambient* and *Heat*, respectively (Fig. 1). *Ambient* and *Fjord* temperatures were almost persistently slightly above the 22-yr temperature average of the Kiel Fjord in winter and spring (black line in Fig. 1; data published in PANGAEA https://doi.org/10.1594/PANGAEA.919186), but decreased to remain slightly below average during the summer months. They substantially exceeded the 22-yr SD of the average in December by 3–4°C for 2 weeks and in June by 2–5°C for 4 weeks (Fig. 1).

The salinity was on average 20.1 PSU (12.8–23.7) in winter (December–February), 14.4 PSU (8.6–19.4) in spring (March–May), and 15.9 PSU (9.7–20.8) in summer (June–August; Supporting Information Fig. S1), following a natural seasonal pattern for this area (Wasmund et al. 2008). Sudden changes in salinity can be explained by weather (e.g., heavy rain or strong mixing events). Salinity in each benthocosm was very similar to salinity measured in the fjord indicating sufficient water exchange, which prevented freshening during rain and increase in salinity during sunny summer days. Oxygen concentration strongly followed the temperature regime, being highest in winter with up to 458 μmol L^{-1} in *Ambient* and lowest in late spring in *Heat* with values as low as 210 μmol L^{-1} (Supporting Information Fig. S2).

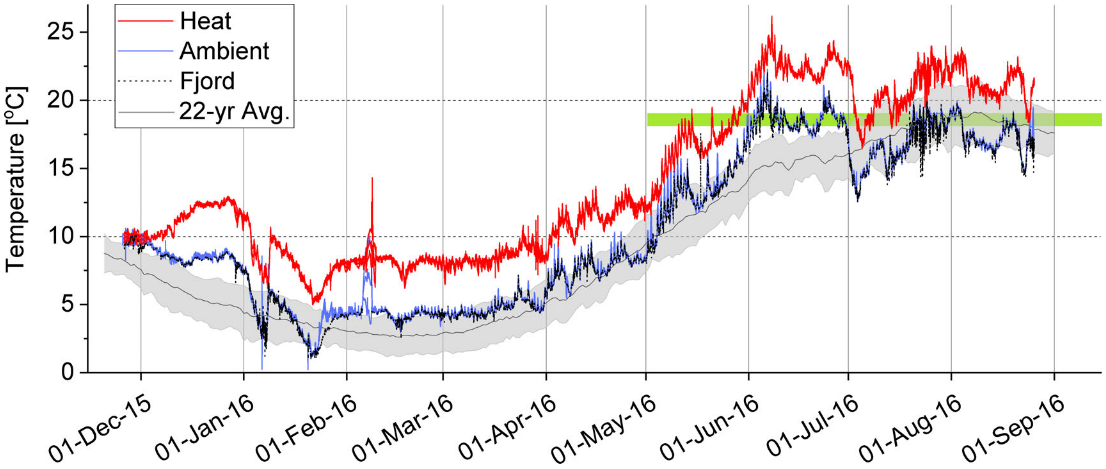

Fig 1. Measured temperature regime of *Ambient* and *Heat* benthocosms (mean of six replicate tanks ± SD [same color]) from December 2015 into August 2016. Measured SST in the Kiel Fjord (1 m depth) during the experiment (Fjord) is illustrated by a dashed black line. Averaged SST of the years 1997 to 2018 in the Kiel Fjord ± SD (gray line and area; data provided by the GEOMAR, Ocean Circulation and Climate Dynamics – Marine Meteorology [www.geomar.de/en/service/weather]). Green bar indicates optimal growth temperature of *Z. marina* in summer (Lee et al. 2007).

Concentrations of NO_2 and NO_3 combined were on average 16.4 μmol L^{-1} between December and March and below 5.2 μmol L^{-1} from April to August), and were similar in *Ambient* and *Heat* (Supporting Information Fig. S3). NH_4 concentrations varied between 1.6 (March) and 6.9 μmol L^{-1} (June) and were slightly higher in *Heat* than in *Ambient* at most sampling points (Supporting Information Fig. S3). PO_4 concentrations were below 0.5 μmol L^{-1} from March to May and between 0.6 and 1.0 μmol L^{-1} in winter and in summer (Supporting Information Fig. S3). The nutrient concentrations along the experiment followed seasonal nutrient concentrations and fluctuations for the studied area, which is largely driven by the activity of phytoplankton (Lennartz et al. 2014).

Z. marina response

The abundance of originally introduced *Z. marina* shoots remained close to 100% over the first 2.5 months of the experiment (beginning of December to mid-February) in *Ambient* and *Heat*, but declined between mid-February and mid-April in *Ambient* (to 91% ± 7.2%; mean ± SE) and, in particular, in *Heat* (to 59.5% ± 9.7%; Fig. 2a). From mid-April to mid-August, shoot abundances decreased further to 61.0% ± 4.0% and to 35.0% ± 4.6% in *Ambient* and *Heat*, respectively (Fig. 2a). The decline in shoot abundance was significantly stronger in *Heat* than in *Ambient* (Fig. 2a; Table 1; Supporting Information Fig. S4).

New shoots occurred in highest numbers in winter (January and February) with a maximum of 16.7% ± 1.9% per month of the originally introduced shoots in *Ambient* and with a maximum of 12.5% ± 10.9% in *Heat* (Fig. 2b). New shoots also occurred throughout the other seasons of the experiment with a second peak registered in *Ambient* in May (13.9 ± 4.1). The number of new shoots was overall significantly higher in *Ambient* than in *Heat* (Fig. 2b; Table 1; Supporting Information Fig. S4).

The total number of sexually reproductive shoots throughout the experiment was 10.4% (0–20.8% per benthocosm) of the originally introduced shoots in *Ambient* and 6.9% (0–16.7%) in *Heat*. The peak of reproduction in *Heat* occurred earlier in the year (~ 1.5 months) than in *Ambient*, with 2.8% ± 1.4% (from introduced plants) registered in *Heat* in the beginning of April and 3.5% ± 1.7% registered in *Ambient* in mid-May (Fig. 2c). Despite an apparent difference in reproduction peak in Fig. 2c, the GLMM and subsequent Chi-square test did not produce a significant result (Table 1). This is due to the overall low percentage of reproductive plants, coupled with a high variability between benthocosms. To counter the overall low numbers (and numerous zeros), we tested the models again with a cumulative approach, meaning that the reproductive plants were added up successively. This produced a significant difference between *Ambient* and *Heat* (Table 1; Supporting Information Fig. S4).

Growth rates (leaf extensions) were repeatedly measured between mid-March, when first growth marks were applied, and in mid-August. While both treatments showed highest rates in June with 4.2 ± 1.5 cm d^{-1} in *Ambient* and 3.2 ± 1.3 cm d^{-1} in *Heat* (Fig. 2d), growth rate dynamics varied significantly between treatments (Table 1). Growth rates of *Heat*-treated shoots were higher than *Ambient* shoots during spring, but lower in summer, and showed an overall lower seasonal response (Fig. 2d; Supporting Information Fig. S4).

The duration for the development of a new leaf (P_L) averaged 10.7 ± 0.4 d for *Ambient* and 16.2 ± 1.1 d for *Heat* treatment. The shortest and longest P_L were measured in June and end-March in *Ambient* treatment, respectively. In *Heat* treatment the shortest P_L occurred in May and the longest at end of March and beginning of August (Fig. 2e). Despite the slight differences between *Ambient* and *Heat*, no overall significant

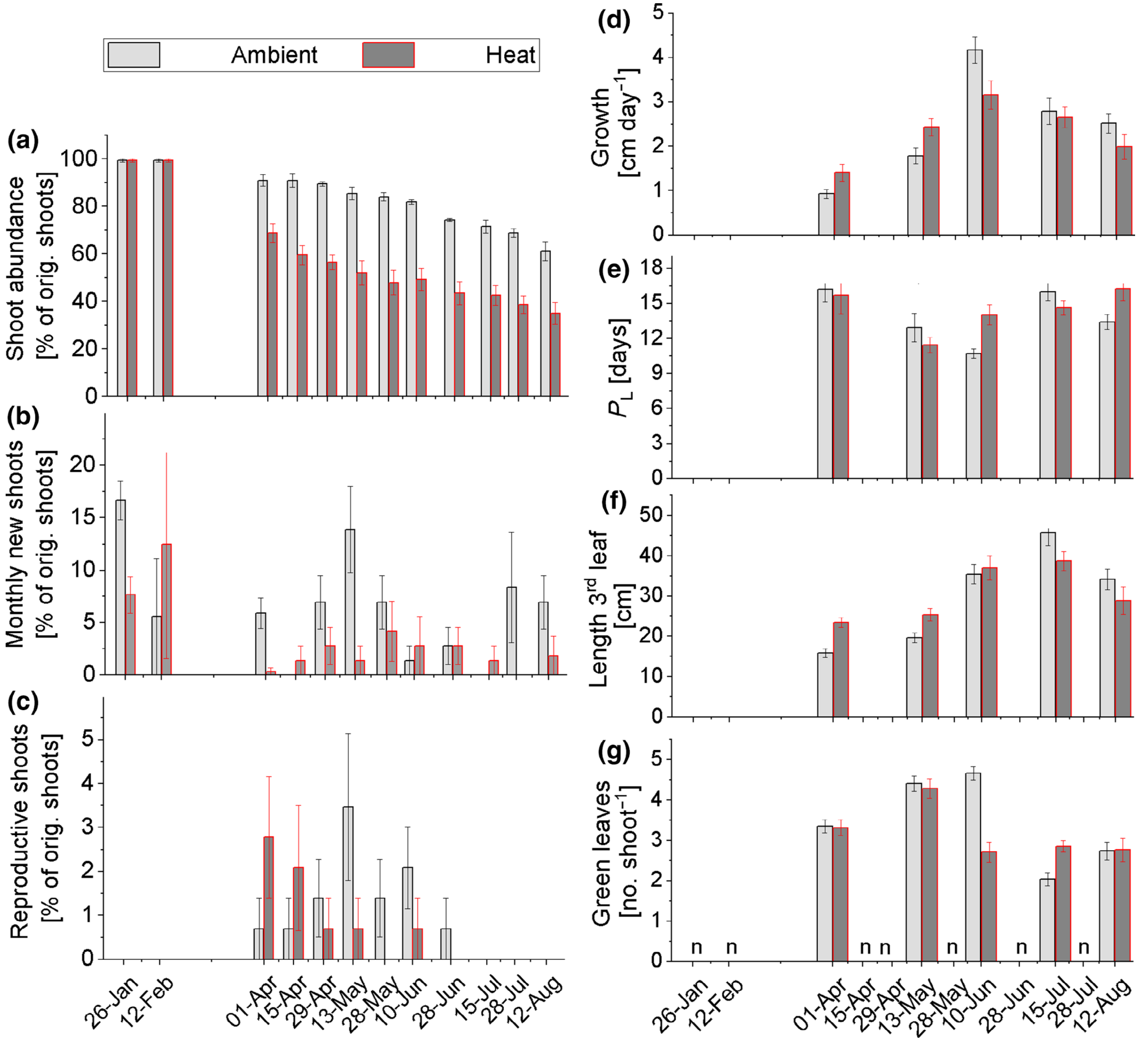

Fig 2. *Z. marina* responses (mean ± SE). Abundance of original shoots (survival, **a**), occurrence of new shoots standardized to month (**b**) and abundance of sexually reproductive shoots (**c**) in relation to the number of original shoots (100%), measured bimonthly between mid/end of January and mid-August 2016, except from mid-February to April. Seagrass growth (leaf extension, **d**), time to grow a new leaf (P_L, **e**), lengths of the 3rd leaf of a shoot (**f**) and number of green leaves per shoot (**g**) were measured monthly between beginning of April and mid-August 2016. *n* = no data available at the given time points. *n* indicated in **g** is also representative for the parameters presented in **d**, **e**, and **f**.

effect of *Heat* on P_L was found (Fig. 2e; Table 1; Supporting Information Fig. S4).

The lengths of the 3rd leaf of a shoot generally followed the pattern of growth rates, although with a short delay (Fig. 2f). Shortest lengths were measured beginning of April, being 15.8 ± 1.1 cm in *Ambient* and 23.5 ± 1.2 cm in *Heat* (Fig. 2f). Longest leaves were measured in July with 45.7 ± 3.1 cm in *Ambient* and 38.8 ± 2.4 cm in *Heat* (Fig. 2f), 1 month after the highest growth rates were recorded (Fig. 2d). Like the growth rates, treatment had a significant effect on the length of the 3rd leaf (Table 1) being higher in *Heat* than in *Ambient* in spring and lower in *Heat* in summer (Fig. 2f; Supporting Information Fig. S4).

The numbers of green leaves per shoot were similarly high in *Heat* and *Ambient* in April and May, but dropped

Table 1. Results of model comparison (Chi-test; "treatment GLMM" vs. "null GLMM"). df = degrees of freedom. Significant results are in bold. Results of biomass directly derived from the GLMM.

Response variable	Chi-square	df	*p*-value
Shoot abundance	470.32	3	<0.001
Monthly new shoots	89.60	3	<0.001
Reproductive shoots	8.92	4	0.063
Reproductive shoots (cumulative)	10.71	3	0.013
Growth	129.39	5	<0.001
P_L (days to produce new leaf)	6.23	3	0.101
Length of 3rd leaf	31.05	3	<0.001
Green leaves	6.20	4	0.185
Chl *a*	3.96	3	0.266
β-Carotene	0.58	3	0.902
Chl *a*/β-carotene	7.32	3	0.062
		t-value	
Biomass below ground		2.45	0.014
Biomass above ground		2.785	0.005

substantially during (*Heat*) and after (*Ambient*) the June heatwave (Figs. 1, 2g). In the *Heat* treatment, the number of green leaves per shoot dropped from 4.2 ± 0.2 in May to 2.7 ± 0.3 in June, and in *Ambient* from 4.7 ± 0.2 to 2.0 ± 0.2 in July (Fig. 2g).

At the end of the experiment, below- and above-ground biomass was almost double and significantly higher in *Ambient* than in *Heat* (Fig. 3; Table 1; Supporting Information Fig. S4), despite similar growth rates throughout the experiment and only slightly (nonsignificantly) longer length of 3rd leaf and equal number of green leaves per shoot in *Ambient* in August (*see* above).

The concentration of the pigments Chl *a* and β-carotene fluctuated largely concomitantly over time with highest concentrations found at the beginning of April and in mid-June (Fig. 4a,b) and an additional peak for β-carotene at the end of July (Fig. 4b). Pigment concentrations were comparatively low in May as well as at the end of the experiment (mid-August). The applied temperature treatment did not have an overall significant effect on pigment concentrations (Table 1; Supporting Information Fig. S5); however, lower Chl *a* (−25.8%) and lower Chl *a* (−20.4%) and β-carotene (−16.6%) were evident in *Heat* compared to *Ambient* in June and July, respectively. The ratio of Chl *a*/β-carotene was similar throughout most of the experiment, but showed a clear depression in July (*Ambient* and *Heat*). Temperature treatment did not have an overall effect on the ratios (Table 1; Supporting Information Fig. S5), but showed lower ratios in June in the *Heat* treatment compared to *Ambient* (Fig. 4c).

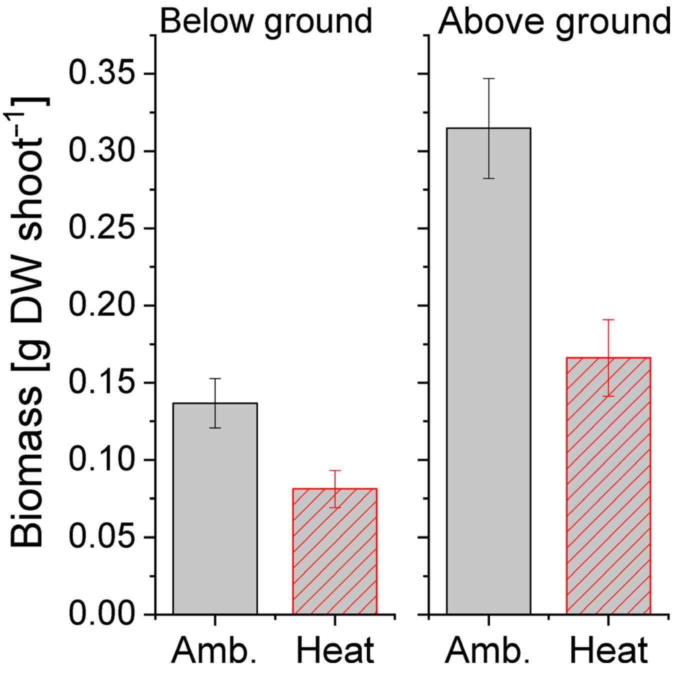

Fig 3. *Zostera marina* above- and below-ground biomass (mean ± SE) presented as dry weight (DW) per shoot.

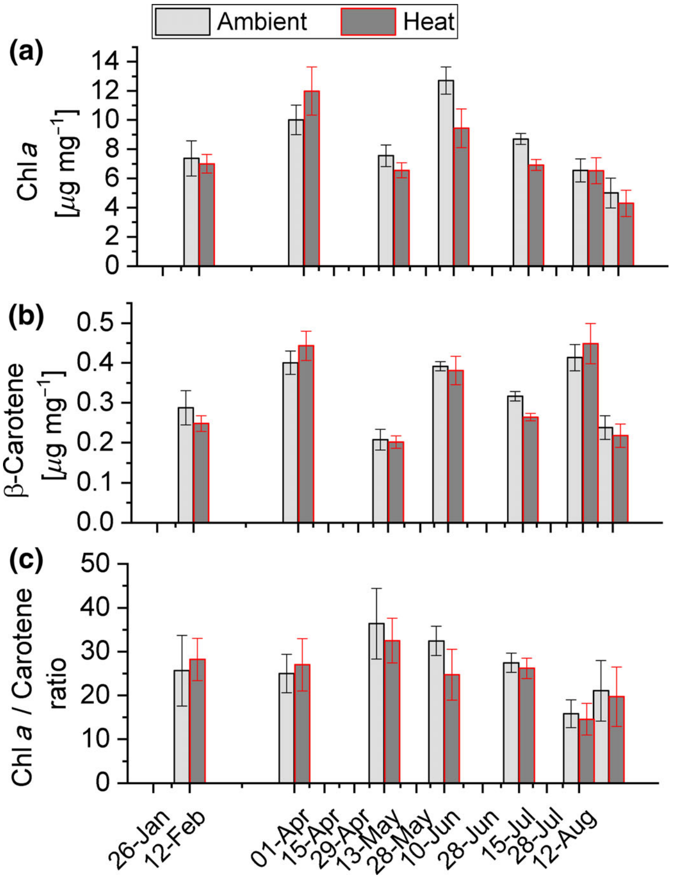

Fig 4. *Zostera marina* leaf pigment concentration (mean ± SE) of Chl *a* (**a**) and β-carotene (**b**) per mg leaf dry weight and the ratio of Chl *a* to β-carotene (**c**) measured monthly between mid-February and mid-August, except in March (no measurement) and August (bimonthly).

Discussion

In contrast to a number of rather short-term manipulation experiments, this study uses a rare experimental approach by investigating the effect of elevated SST on the temperate seagrass *Z. marina* across three seasons. The goal was to assess the effect of overall increasing SST on *Z. marina*, in particular in winter and spring, when elevated SST may not evoke a heat stress response per se, but may rather cause a loss of biomass that entails detrimental effects in the following months. The study was conducted using outdoor mesocosms providing near-natural conditions, including naturally occurring fluctuations in temperature, light, and nutrient supply. Moreover, although not further investigated here, the experiment included associated organisms that may have modulated the responses of seagrass through ecological interactions, for example, through the occurrence of epiphytes and grazers (Paiva et al. 2021). An added value of this study is that the results can be placed into an environmental context using a 22-yr SST time series, which is rare in ex situ or in situ experiments, but of particular value in highly dynamic environments (i.e., high interannual, seasonal and day-to-day variability). Following this, the *Ambient* temperature treatment encompassed two periods of abnormally high SST (marine heatwaves) that occurred naturally during the time of experimentation—in December 2015 and in June 2016. The *Heat* treatment experienced a stronger abnormality of high temperatures between December 2015 and June 2016 than between July and August 2016. This experimental approach together with the background data available can provide a more realistic outlook into how overall increasing SST (in contrast to short-term heatwaves; e.g., *see* Saha et al. 2020) may affect an important foundation species of temperate coastal ecosystems, namely *Z. marina* seagrass beds.

The main results of this study are (1) a high survival of shoots in winter independent of temperature, which is (2) followed by an advanced onset of sexual reproduction and a concomitantly high mortality under *Heat* conditions in spring. After completion of sexual reproduction of both *Ambient* and *Heat*-treated *Z. marina*, (3) growth characteristics, pigmentation, and survival were largely comparable between treatments (May to August), while the production of new shoots was low under *Heat* conditions. (4) A comparatively high mortality in *Ambient* shoots occurred during summer, after an abnormally warm June and (5) above- and below-ground biomasses were reduced by ~ 50% in *Heat*-treated compared to *Ambient Z. marina* at the end of summer (end-August). We attribute these findings mainly to the dynamics of the energy budget of *Z. marina*.

Z. marina of mid-latitude regions accumulates most of its energy reserves from late spring to mid-autumn (Vichkovitten et al. 2007), when high SST and high-light conditions (long days and high light intensity) lead to increased primary production (Lee et al. 2007). Energy reserves are then gradually depleted during low-light winter conditions, when demand (respiration) is higher than the energy gain, reaching a minimum in early spring (Vichkovitten et al. 2007). The results of our study indicate that despite temperature-driven intensification of respiration (Staehr and Borum 2011) the plant's energy stores were sufficient to meet the increased metabolic requirements during the warm winter. However, toward the end of winter, energy reserves in *Heat*-treated plants most likely reached critical levels, which could not be compensated by light-limited photosynthetic rates (Olesen and Sand-Jensen 1993). Consequently, several shoots died (~ 30% by end-March), while others invested in an early onset of sexual reproduction, which may likely be a stress response (Kazan and Lyons 2016).

The typical flowering season of *Z. marina* in the Baltic Sea is between March and August, with a peak in flowering around May (Olesen 1999). Advanced flowering in plants can be triggered by a variety of abiotic and biotic factors, such as heat and salinity stress, droughts, and pathogen infections (Kazan and Lyons 2016). Naturally, the onset of flowering in many plants of mid-latitude regions is triggered by photoperiod and temperature (Ausin et al. 2005). A meta-analysis conducted with hundreds of terrestrial plants in the UK and the East coast of the United States showed an early onset of flowering during warm winters and/or springs in ~ 80% of plants, including a number of grass species (Fitter and Fitter 2002; Cook et al. 2012), which demonstrates a strong temperature-dependency of flowering traits. Much less evidence is available for SST effects on flowering in marine plants.

Few laboratory studies on seagrass species found a positive relationship between temperature and the amount of flowering plants; however, they did not investigate the timing of flowering (*Halophila engelmanni*, McMillan 1976; *Z. marina*, Cock 1981). At a first glance, our results imply that flowering of *Z. marina* is strongly temperature driven. However, in contrast to our expectations it is evident that flowering in *Heat*-treated shoots peaked at a lower temperature (< 10°C) in comparison to the shoots at *Ambient* (> 10°C). Therefore, a more likely explanation for the early onset of flowering might be an ultimate stress response stimulated by critically reduced energy reserves. This is corroborated by a recent study, where the temperate seagrass species *Posidonia oceanica* flowered unexpectedly during a summer heat-stress experiment, 1 month before its normal flowering season (Marín-Guirao et al. 2019). The authors concluded that this was a heat-stress response triggered by a depletion of energy reserves (sugars and starch), and provided transcriptomic support for a link between sugar metabolism and flowering (Marín-Guirao et al. 2019). This link is well known for terrestrial plants where sugar has been found to play a central role in signaling a stress condition ultimately leading to flowering (Coneva et al. 2012; Moghaddam and Van den Ende 2013; Kazan and Lyons 2016). As most stress responses lead to a faster consumption of energy reserves, it seems reasonable that the depletion of energy reserves induced an early onset of flowering in *Heat*-treated *Z. marina* of our experiment.

The number of sexually reproductive shoots found in this study (10.4% in *Ambient* and 6.9% in *Heat*) is comparable to observations on *Z. marina* in the subtidal of other mid-latitude regions (North-West Atlantic: 2–12%; Phillips et al. 1983 and 11–19%: Silberhorn et al. 1983; North Sea: ~ 10%, Potouroglou et al. 2014). It needs to be considered, however, that a lack of sampling between mid-February and end of March may have resulted in an underestimation of flowering shoots in the *Heat* treatment. Indeed, increased investment in sexual reproduction of *Z. marina* growing under rather challenging conditions is well documented (e.g., in the intertidal; Keddy 1987; Phillips et al. 1983; Cabaco and Santos 2012), and, importantly, this mechanism seems to be plastic (based on transplantation experiments; Keddy 1987). Alternatively, the exceptionally warm winter temperature in the *Heat* treatment of our experiment (up to > 7°C above the 22-yr average) may have caused the mortality of several shoots before being able to reproduce. Vegetative in contrast to sexual reproduction is considered to be the dominating mode of reproduction in perennial *Z. marina* stands (Olesen and Sand-Jensen 1994; Boese et al. 2009). New shoots were evident in all seasons, but were highest in winter in both treatments. This is consistent with a recent observation of *Z. marina* at the donor site of this experiment, where a rather high shoot production was found in the winter of 2019/2020 (F. Weinberger pers. comm.). *Ambient* shoots experienced a smaller second reproductive peak in May, which is in line with the previously identified peak of vegetative reproduction of *Z. marina* in the Danish Baltic Sea (May/June; Olesen and Sand-Jensen 1994). In contrast, the overall low shoot production in *Heat*-treated plants after the winter peak is likely the consequence of high mortality of original shoots in spring and a general shortage of energy in surviving shoots.

Z. marina growth generally followed the expected trend with SST in both treatments, with highest performance around 18°C. P_L was fastest in June in *Ambient* (10 d at 18–20°C) and fastest in May in *Heat* (11 d at 16–18°C) being comparable to rates previously reported (e.g., 10 d at 20°C in Denmark; Beca-Carretero et al. 2018). Growth rates (leaf elongation) were higher in *Heat*-treated shoots than in *Ambient*-shoots before reaching their peak in June, when the particularly high temperature in the *Heat*-treatment reversed this pattern (*Heat*: 3.1 cm d^{-1}; *Ambient*: 4.1 cm d^{-1}). The growth peak in our study seems early and growth rates in July and August rather small, compared to other studies. For example, a peak in growth rates of 5–6 cm d^{-1} was found in July in the Western Baltic Sea (Worm and Reusch 2000). However, a similar study as presented here, using the same mesocosm system (Saha et al. 2020), found a peak of *Ambient* growth rates in the beginning of August (18–20°C) with rates of 2.8 cm d^{-1} and were as low as ~ 2 cm d^{-1} at the end of May (15–16°C; Y. Sawall unpubl.). The early peak of growth performance in our study can therefore be explained by the exceptionally high SST in June, which represented the warmest period of the entire 9-months experiment. Similar growth rates in *Heat* and *Ambient* in July and August are likely due to a rather cool summer (*Ambient* was below the 22-yr average SST), representing SSTs just above optimum in *Heat* and just below summer optimum in *Ambient* (Fig. 1).

The exceptionally high SST in June may also be the reason for lower Chl *a* concentrations in *Heat* compared to *Ambient* in June, when thermal stress may have led to photodamage (Guo et al. 2006). Apart from that, however, treatment-driven differences in pigment concentrations were small or absent, which is in line with a previous heat stress experiment conducted on seagrass (*Zostera muelleri*) in SE-Australia (York et al. 2013). Here, Chl *a* was unaltered by the temperature treatment and photoprotective pigments were upregulated only at the suboptimal (not yet lethal) upper temperature limit (York et al. 2013). Keeping in mind that β-carotene measured in our study is only a precursor of photoprotective pigments and not a photoprotective pigment itself, we can only speculate that our temperature treatment had no effect on the seagrass' ability for photoprotection.

While a strong selection for heat-resistant shoots occurred already early in the year in the *Heat* treatment (high mortality in early spring), a rather high mortality occurred at *Ambient* after the June heatwave. This may be ascribed to a cumulative negative effect of an abnormally warm winter and early summer that led to a tipping point in plant performance. This finding is in contrast to a previous study where the authors tested the effect of a single and of multiple short summer heatwaves on *Z. marina* performance (Saha et al. 2020). Here, heatwave events had a rather small effect on *Z. marina* based on the fact that only leaf elongation and P_L were compromised after three consecutive short-term summer heatwaves, while the other five response parameters measured (related to metabolic rates and anti-pathogen defense) did not show any effect (Saha et al. 2020). In the same study, shoot mortality was low throughout the experimental phase (May to August 2015) with < 5% (Y. Sawall unpubl.). This comparison provides indication for a stronger temperature sensitivity of *Z. marina* earlier in the year, which is also supported by a previous study (Staehr and Borum 2011). Another factor that warrants a brief discussion in this context is the timing of plant transplantation from the field to the mesocosms. A plant-transplantation always entails a partial loss of roots and rhizomes. This loss may be particularly challenging in November as the low-productivity winter starts. Therefore, we cannot rule out the possibility that the timing of our transplantation may have in parts contributed to the mortality of shoots, in both *Heat* and *Ambient* conditions. A transplantation earlier in the year (e.g., during spring/early summer, late summer) may therefore be recommended for future experiments (short-term or trans-seasonal) conducted at latitudes with low-light winters (e.g., > 50°N).

Surviving shoots in the *Heat*-treatment had only ~ 50% of the biomass of that of *Ambient* shoots, despite overall comparable growth rates throughout the experiment and similar lengths and number of green leaves at the end of the experiment. The difference in above-ground biomass may derive from reduced leaf width or leaf thickness in *Heat*, both of which were not quantified. Previous studies have shown that changes in light and nutrients can alter seagrass leaf thickness and breakability (Collier et al. 2012; La Nafie et al. 2013), and increasing temperature was shown to reduce leaf thickness in some terrestrial plants (Chabot and Chabot 1977; Boese and Huner 1990). The reduced biomass of rhizomes and roots in *Heat* vs. *Ambient* plants is more plausible. In contrast to leaves, rhizomes and roots have a longer lifetime and in particular the thick and fleshy rhizomes are the main compartments for energy storage of seagrass plants (Fourqurean and Zieman 1991; Vichkovitten et al. 2007). The reduced biomass is therefore likely the integrated result of a number of processes that were potentially down- or upregulated as a consequence of the applied heat stress. These processes may include (1) lower productivity evident in reduced growth, reduced number of green leaves, increased P_L, and reduced Chl *a* during the June heatwave; (2) higher respiration rates throughout the experimental period; and (3) higher investment of energy into heat-stress defense and repair from June onward. It seems unlikely that heat-stressed plants are capable to restock their energy reserves in time before the low-productivity winter arrives, which could lead to further loss of seagrass, under abnormally high SST, or even under ambient conditions as a lag effect. Considering again that the summer temperatures of our experiment were comparatively low, the loss of energy reserves or shoots would have likely been exacerbated during normal summer temperatures.

In summary, our results show that an abnormally warm winter and spring can severely affect *Z. marina* populations, possibly leading to equal or even more detrimental effects than summer heatwaves. Although we cannot exclude the possibility that transplantation in November may have been particularly challenging and thereby exacerbated the stress response of *Heat*-treated shoots in spring, the study clearly demonstrates that warm winters can lead to high mortality and advanced sexual reproduction as a consequence of dramatic declines in seagrass energy storage. Whether a stress-induced advanced flowering suppresses or increases reproductive success can depend on a variety of factors and remains to be investigated. A recent study suggests that early flowering could be an adaptive response to changing environmental conditions through epigenetic modification (Marín-Guirao et al. 2019), yet ignoring the viability of seeds. Interestingly, *Heat*-treated shoots that survived the early spring die-off in the present study were able to perform fairly well during the extraordinary warm late spring and throughout summer. However, profound reductions in biomass clearly indicate the cumulative and lasting effect of consistently elevated SSTs, which may eventually lead to the starvation of seagrass populations, in particular if summer SST are higher than experienced during the rather cool summer of this experiment. These negative implications induced by increasing SSTs may be overcome by an increased effort in sexual reproduction leading to higher genotypic diversity in seagrass populations, considering that genetic diversity increases seagrass resilience (Hughes and Stachowicz 2004; Reusch et al. 2005; Ehlers et al. 2008). Alternatively, epigenetic modifications may equip seagrass with a higher thermal tolerance (Verhoeven and Preite 2014; Latzel et al. 2016; Lämke and Bäurle 2017), required that available energy reserves allow these investments. While our study provides a rare but important insight into the effect of trans-seasonal warming effects on seagrass performance, multi-year and cross-generation experiments are required to assess whether seagrass populations are able to keep up with the pace of on-going global warming trends and their temporal extremes that are expected to increase in frequency and amplitude over the next few decades.

Data Availability Statement

All data will be available at PANGAEA database:
https://pangaea.de/10.1594/PANGAEA.937382
https://pangaea.de/10.1594/PANGAEA.937383
https://pangaea.de/10.1594/PANGAEA.937384
https://pangaea.de/10.1594/PANGAEA.937386
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https://pangaea.de/10.1594/PANGAEA.937393
https://pangaea.de/10.1594/PANGAEA.937394

[Correction added on 30 November 2021, after first online publication: The incorrect DOI URLs of PANGAEA has been removed]

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Conflict of Interest

None declared.

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