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High temperatures cause reduced growth, plant death and metabolic changes in eelgrass *Zostera marina*

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ABSTRACT: We investigated temperature effects on eelgrass *Zostera marina* L. growing close to its southern distribution limit along the eastern coast of North America in Virginia, USA. We combined growth and survival experiments with microelectrode measurements of internal meristematic oxygen and analyses of metabolic compounds. Eelgrass shoots were grown at 3 different temperatures (22, 26 and 30°C) and field equivalent light levels (23% of sea surface insolation) for 28 d while water column oxygen concentration was kept at air saturation. Meristematic oxygen concentrations did not vary significantly with temperature, and meristems maintained a relatively high oxygen concentration (average: 38% air saturation) during dark hours. Despite high meristematic oxygen concentrations, shoots growing at 30°C exhibited increased mortality, reduced growth and reduced leaf production compared to shoots growing at 22 and 26°C. The leaf metabolome was significantly altered at 30°C, indicating an increase of reactive oxygen species. In addition, total nitrogen and metabolites related to the nitrogen cycle (amino acids, urea and γ-aminobutyric acid [GABA]) were low in the heat-stressed shoots, whereas soluble sugars increased. In conclusion, high temperature (30°C) has strong negative effects on eelgrass in the lower Chesapeake Bay, affecting growth, tissue integrity, nitrogen metabolism and protein/enzyme synthesis. Future global warming may likely deteriorate populations of eelgrass at its present southern distribution limit.

KEY WORDS: *Zostera marina* · Temperature stress · Growth · Metabolomics · Climate change

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INTRODUCTION

Seagrass populations have declined globally for decades owing to physical disturbance and eutrophication, and in many regions losses have been compounded by increasing global temperatures (Orth et al. 2006). Eelgrass *Zostera marina* L., the most widespread seagrass in the Northern Hemisphere, has also undergone marked declines in distribution and abundance (Short & Wyllie-Echeverria 1996, Orth et al. 2010). Even though eelgrass is exposed to a wide range of temperatures across its range from arctic to warm temperate regions (den Hartog 1970), the annual rate at which the temperature is rising along with unusually high summer temperatures during heatwaves may affect long-term survival. Eelgrass populations growing at the southern distribution limit are likely the most vulnerable to future warming, since they are expected to have reached the maximal adaptation to heat within the eelgrass genome (Moore & Jarvis 2008, Moore et al. 2012). Eelgrass populations growing in colder regions may also be affected when exposed to unusually high temperatures (Bergmann et al. 2010, Franssen et al. 2011, Gu et al.

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2012, Beca-Carretero et al. 2018). In addition, synergetic or additive effects of temperature and other stressors, such as low salinity, low light and high nutrient load, have been experimentally documented (Salo & Pedersen 2014, Moreno-Marín et al. 2018).

During summer heat waves, sudden die-offs are regularly observed, particularly after periods with calm and exceptionally warm weather (Plus et al. 2003, Greve et al. 2005, Moore & Jarvis 2008). Healthy looking shoots are then found detached from the rhizome at the intercalary meristematic region (Greve et al. 2005). This pattern suggests that the intercalary meristem is especially vulnerable to anoxia due to a high oxygen consumption rate (Pulido & Borum 2010). Oxygen supply can be compromised when high temperature causes increased respiration and lower oxygen solubility (Raun & Borum 2013). This combination may result in insufficient energy production by anaerobic metabolism and tissue acidification which may induce shoot death within 8 h (Pulido & Borum 2010). Tissue anoxia is often combined with intrusion of potentially toxic sulfide, which is formed in the sediment and enters the shoots through the roots during night when water column oxygen levels drop (Borum et al. 2005, Holmer & Hasler-Sheetal 2014, Brodersen et al. 2017). If the water column is well oxygenated, the shoots prevent sulfide intrusion by transferring oxygen from the water column through the well-developed aerenchyma to the rhizome and roots (Pedersen et al. 2004, Borum et al. 2005). However, water column oxygen levels in eelgrass beds can regularly reach hypoxia and even anoxia during warm summer nights (Moore 2004) due to high levels of benthic respiration and low levels of water movement.

While negative effects of anoxia are partly induced by high temperature, we have little knowledge of direct temperature effects on seagrasses. It is possible that heat is stressing and damaging the shoots and causing sudden die-backs (Zimmerman et al. 1989, Nejrup & Pedersen 2008). To assess various stress effects, metabolomics analyses have recently been used on seagrasses (Gu et al. 2012, Hasler-Sheetal et al. 2015, 2016, Kumar et al. 2016). Metabolomics gives a snapshot of the composition of many metabolites, offering a tool to evaluate phenotypic responses of organisms to environmental conditions (Kumar et al. 2016). During periods of environmental stress, plants typically alter their metabolic composition as a response to the stressor (Arbona et al. 2013). Heat stress is typically related to destabilization of proteins, membranes, RNA species and the cytoskeleton, while enzymatic reactions are also altered (Mittler et al. 2012).

In this study, we investigated the effects of increasing temperature on eelgrass populations located close to their southern distribution limit along the eastern coast of North America and attempted to detect some of the mechanisms that are activated during heat stress. Furthermore, we separated the effects of anoxia and temperature by keeping the water column well oxygenated (100% air saturation) while exposing shoots to temperatures from 22 to 30°C. We combined growth and survival measurements with advanced microelectrode measurements and analyses of metabolic compounds to couple the phenotypic response of the shoots to the metabolic mechanisms. We hypothesized that rising temperature will (1) decrease shoot growth and survival, (2) lower tissue oxygen concentrations during dark hours due to increased respiration and (3) reprogram the metabolic network, which may reveal details of the nature of heat stress.

MATERIALS AND METHODS

Plant material and experimental setup

In late August 2015, sediment cores containing eelgrass shoots were sampled from shallow water surrounding Goodwin Island, Virginia, USA (37° 13′ 1″ N, 76° 23′ 19″ W). At the time of sampling, shoots had exhibited a dieback after the warm summer and grew in sparse patches. The patches from which the cores were taken were selected by snorkeling around the area to find shoot material with similar shoot density. Despite being close to the southern distribution limit of eelgrass, eelgrass stands in this area are known to have high sexual reproduction and high genetic diversity (Reynolds et al. 2012). Nine cores were retrieved from different patches using a Plexiglas corer with a diameter of 25 cm to a sediment depth of 20 cm, and transferred to plastic pots of the same diameter. The deep sediment and relatively large core diameter ensured intact root systems and only a few damaged shoots. The individual pots with undisturbed shoots and sediments were kept in buckets covered with seawater and transported to the laboratory within 1–2 h.

The water temperature at the collection time was 28°C, and the salinity was 20.6 psu. The cores were first transferred to a tank (2.7 × 1.4 m and 0.6 m deep) with circulating seawater at a temperature of 26°C and a salinity of 20 psu in a 0.5 m water column. The pots with intact shoots in their sediments were allowed to acclimate for 3 d before they were distrib-

uted into 3 additional tanks similar to the first tank. Each tank received 3 randomly picked pots, and temperatures were increased, decreased or kept unchanged over 5 d (to allow for acclimation) until experimental temperatures of 22, 26 and 30°C were reached. The shoots were exposed to the different temperature treatments for 28 d.

The temperature in each tank was controlled by circulating the water through an external air-cooled heat pump (Delta Star, Aqualogic). At the same time, the water was aerated, filtered (Tarpon Sand Filter, Lifegard Aquatics) and illuminated by UV light (SMART UV Sterilizer, Pentair Aquatic Eco-systems) to air saturate the water and minimize growth of bacteria and phytoplankton. The tanks were placed in a greenhouse with fans to help stabilize temperatures. Temperatures were logged continuously by HOBO loggers (Onset Computers).

Light followed the natural diel cycle as the sun was the only light source. Light levels were continuously recorded both outside and inside the greenhouse (LI-192 quantum sensors, LI-COR Biosciences). Light was reduced to 22.9% of full incident light using shades at the greenhouse roof. The light environment at the sampling site was calculated as in Moore et al. (2014) using the continuously recorded water depth (YSI 6600 V2 Multi-parameter Water Quality Sonde, YSI) and light attenuation measurements (LI-192 quantum sensor, LI-COR Biosciences) at the Goodwin Island sampling site (www.vecos.org) and a nearby Taskinas Creek, VA, meteorological station (37° 24′ 55“, 76° 42′ 53“). The mean light attenuation coefficient in the water of 1.66 m^{-1} was applied. The preceding monthly average percentage of light reduction at the sediment surface at the sampling site at 0.9 m mean sea level relative to full incident light was determined to be 22.4%. Thus, the average light conditions in the experiment and in the field were similar.

During the experiments, water evaporated from the tanks, and salinity was allowed to rise to 22 psu before the tanks were refilled with tap water and reset at 20 psu. Temperature, conductivity, salinity, dissolved oxygen and pH were measured using a YSI 6600 V2 minisonde before and after tanks were re-filled and also 3–6 times every week to make sure that conditions remained relatively constant over time.

Shoot response parameters

At the start of the experiment, the number of shoots in each pot was counted and 5 randomly chosen shoots were tagged by piercing a needle through the leaves immediately above the sheath of the oldest, fully grown leaf. After 2 wk, i.e. halfway through the experiment, shoots were recounted and leaf growth measured by the displacement of leaf length relative to the mark on the oldest, non-growing leaf (Zieman 1974). Leaf growth was calculated as increase in leaf area by multiplying increase in length by leaf width since treatment also affected leaf width of newly formed leaves. New leaves without marks were assumed to have grown from a hidden position corresponding to half of the length of old leaf sheath hiding them at the time of tagging (Sand-Jensen 1975). New shoots were tagged and calculations repeated at the end of the experiment. Rhizome growth was estimated by measuring the length of the 4 youngest rhizome segments on 3 randomly chosen shoots in each pot at the end of the experiment, when shoots were harvested.

Meristematic oxygen

Diel changes in oxygen concentration of the intercalary meristem (the meristem located between rhizomes and leaves) were recorded during the first 2 weeks (Days 6, 7 and 8) and the last 2 weeks (Days 20, 21 and 22) of the experiment in 1 shoot from each pot during 3 successive days using Clark-type microelectrodes (OX-25, Unisense). The randomly chosen shoots were carefully tied to wooden sticks inserted in the sediment next to the shoot with a thin wire to prevent movement of the meristematic region and prevent breaking the electrode while inserted in the shoot meristem. The meristematic region was uncovered from the sediment by gently removing sediment with a small brush. The microelectrode was carefully manipulated 1000 µm into the meristem using a micromanipulator (Unisense) attached to an aluminum bar firmly bonded to a cinder block. After inserting the electrode, the meristem was again covered with a 5 mm thick sediment layer. To account for differences in light climate between days and differences among specimens, 3 different microelectrodes measured full light:dark cycles in 3 shoots from 1 pot from each treatment, simultaneously. The electrodes were then moved to shoots in 3 other pots from the 3 temperatures for the next cycle and then repeated for the 3 remaining pots, resulting in diel changes in meristematic oxygen from 9 different shoots during 3 d. The microelectrodes were connected to picoammeters (Unisense) and the signal recorded on a laptop computer using the software program PicoLog (Pico Technology). Electrodes were calibrated at 0 and 100% air

saturation at each of the 3 temperatures before and after each measuring cycle. Periods of noise in electrode signals were excluded from the final dataset.

Chlorophyll *a*, total nitrogen and total carbon

Chlorophyll *a* (chl *a*) content was measured on the second youngest leaf of 1 randomly selected shoot from each pot at the end of the experiment using the method of Dennison (1990). The content was normalized to leaf area by analyzing photos of the leaves using the software ImageJ (Schneider et al. 2012).

Freeze-dried and homogenized samples were analyzed for total carbon (TC) and total nitrogen (TN) content using an elemental analysis isotope ratio mass spectrometer (EA-IRMS; Delta V Advantage Isotope Ratio MS with Thermo Scientific EA) as previously described by Holmer et al. (2017).

Metabolomics

Plant tissue was collected for metabolomic analyses at the end of the experiment. The 9 pots were harvested in random order and 3 shoots from each pot were gently uprooted and rinsed in demineralized water, and leaves were carefully cleaned of epiphytes or other non-eelgrass material. The leaves were snap-frozen in liquid N to preserve metabolites, and samples were stored at −80°C until processing. Metabolites were extracted and analyzed as previously described by Hasler-Sheetal et al. (2015, 2016) with slight modifications. In brief, the effect of temperature on eelgrass was compared using analysis of variance (ANOVA). To graphically visualize the data, we used heat maps illustrating Ward clustering of the Euclidian distances among the treatment groups and metabolites, respectively (for a detailed methods description, see Text S1 in the Supplement at www.int-res.com/articles/suppl/m604p121_supp.pdf).

Statistics

The experimental design was a repeated measures ANOVA with temperature as the fixed factor and time as the random factor. Response parameters measured during and at termination of the experiment (i.e. change in shoot density, leaf growth, leaf production and leaf number) were therefore tested by repeated measures 2-way ANOVA. Response parameters measured only at experiment termination (i.e. internode length and leaf TN, TC and chl *a*) were analyzed by ordinary 1- or 2-way ANOVA. Before performing ANOVAs, data were tested for equal variance by using the Brown-Forsythe test, and normality of distributions was analyzed on residuals by using the D'Agostino and Pearson normality test. All statistics were calculated using GraphPad Prism version 7.03. A significance level of $\alpha = 0.05$ was applied in all analyses.

RESULTS

Growth and survival

Shoot density declined significantly with increasing temperature (Fig. 1A; repeated measures ANOVA, $p < 0.05$, see Table 1 for details on ANOVA tests). The effect of temperature was strongest in the

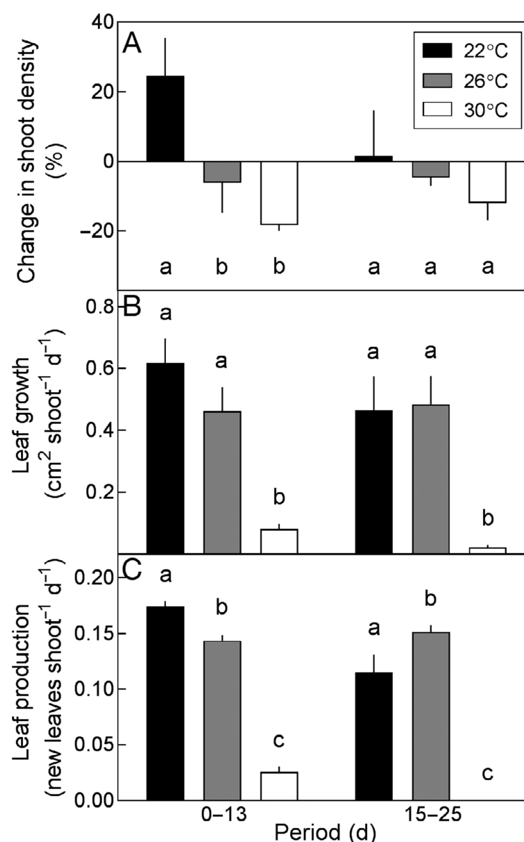

Fig. 1. Eelgrass shoot and leaf growth during the first period (Days 0–13) and second period (Days 15–25) of the experiment at the 3 experimental temperatures (22, 26 and 30°C). (A) Change in shoot density (%). (B) Leaf growth (cm^2 shoot^{-1} d^{-1}). (C) Leaf production (new leaves shoot^{-1} d^{-1}). Mean values ± SE (N = 3). Different letters show significant differences among means within each period (repeated measures ANOVA and Tukey's multiple comparisons test, $p < 0.05$)

Table 1. Results from ANOVAs on eelgrass response parameters. Significant results are shown in **bold**

Response	Source of variation	df	MS	*F*	p
Repeated measures ANOVA					
Change in shoot density	Temperature	2, 6	1240.0	8.78	**0.017**
	Period (time)	1, 6	117.3	0.46	0.521
	Interaction	2, 6	366.5	1.45	0.306
Leaf growth	Temperature	2, 6	0.42	15.52	**0.004**
	Period (time)	1, 6	0.02	3.11	0.128
	Interaction	2, 6	0.01	1.99	0.217
Leaf production	Temperature	2, 6	0.04	176.2	**<0.001**
	Period (time)	1, 6	3.0×10^{-3}	17.32	**0.006**
	Interaction	2, 6	1.7×10^{-3}	9.89	**0.013**
Leaf number shoot^{-1}	Temperature	2, 6	6.09	44.36	**<0.001**
	Period (time)	1, 6	0.10	0.47	0.519
	Interaction	2, 6	0.27	1.25	0.351
Two-way ANOVA					
Internode length	Temperature	2, 24	22.3	29.16	**<0.001**
	Internode number	3, 24	8.09	10.57	**<0.001**
	Interaction	6, 24	1.17	1.53	0.210
One-way ANOVA					
Leaf total nitrogen	Temperature	2, 6	2.24	292.0	**<0.001**
Leaf total carbon	Temperature	2, 6	4.32	59.28	**0.001**
Leaf chl *a*	Temperature	2, 6	20.7	3.83	0.085

first 2 wk (Days 0–13) of the experiment. Shoot density increased at 22°C but declined at 26 and 30°C during the first 2 wk of treatment. The same pattern emerged during the second period (Days 15–25) of the experiment, but differences were not significant. The average shoot density remained largely unchanged at 22°C during the second period, while shoots tended to disappear and mortality increased with increasing temperature at 26 and 30°C. Relative changes in shoot density during this 4 wk experiment are shown in Fig. 2, together with literature data from large-scale field observations of changes in eelgrass cover during monthly intervals as a function of water temperature from Moore et al. (2014). The common trend indicates that shoots in the present experiment reacted to temperature in the same way as shoots in the field.

Shoots growing at 30°C had significantly lower leaf growth compared to those at 22°C and 26°C (Fig. 1B). In the first period (Days 0–13), leaf growth at 30°C was 0.08 ± 0.02 cm^2 shoot^{-1} d^{-1} (mean ± SE, N = 3) and in the second period (Days 15–25), the rate had dropped to 0.02 ± 0.01 cm^2 shoot^{-1} d^{-1}. Leaf growth in the 2 cooler treatments (0.31–0.74 cm^2 shoot^{-1} d^{-1}) was not significantly different from each other in either the first or second period of the experiment (repeated measures ANOVA; Tukey's multiple comparisons test, $p < 0.01$). Formation of new leaves followed the same pattern as leaf growth, being higher at the 2 cooler temperatures (~1 new leaf every week) compared to the 30°C treatment (Fig. 1C). Nevertheless, leaf production at 22°C was significantly lower than at 26°C in the second period, resulting in a significant interaction between time and temperature. This generally high leaf production led to a significantly higher leaf number per shoot at 22 and 26°C (Fig. 3) compared to shoots at 30°C. The 2 cooler temperatures had 4.6–5.8 leaves shoot^{-1} at the end of the experiment while the shoots in the 30°C treatment only had 3.0–3.8 leaves.

Rhizome internode length differed among treatment temperatures at the end of the experiment (Fig. 4). Young internodes were significantly longer than older internodes at 22 and 26°C as growth increased from the field conditions and was stimulated compared to shoots at 30°C. Since 1 internode is created for every new leaf formed (Sand-Jensen 1975), the 4 measured internodes in the 22 and 26°C treatments were produced during the 4 wk

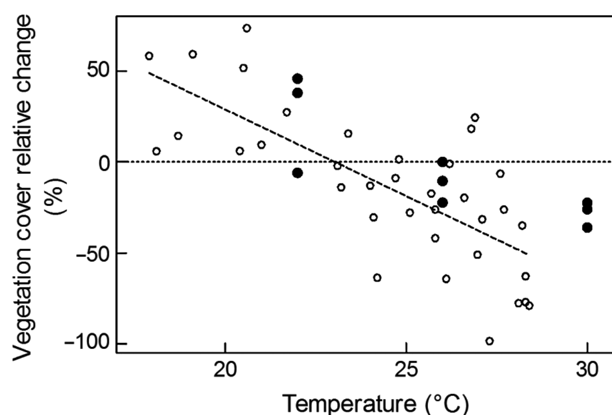

Fig. 2. Relative changes in cover of eelgrass in Goodwin Island, Chesapeake Bay, USA, as a function of mean water temperatures during intervals of 1 mo (open circles) showing temperature range of 0 net expansion at 23.0°C (data from Moore et al. 2014; regression analysis, $p < 0.001$), compared to eelgrass relative changes in shoot density during the 4 wk experiment at the 3 experimental temperatures (22, 26 and 30°C; filled circles)

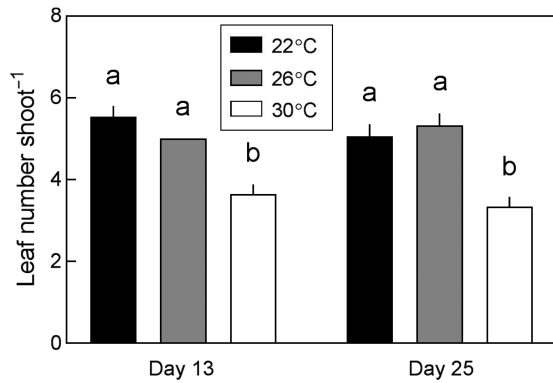

Fig. 3. Total number of eelgrass leaves shoot^{-1} on Days 13 and 25 at the 3 experimental temperatures (22, 26 and 30°C). Mean values ± SE (N = 3). Different letters show significant differences among means within each period (repeated measures ANOVA and Tukey's multiple comparisons test, $p < 0.01$)

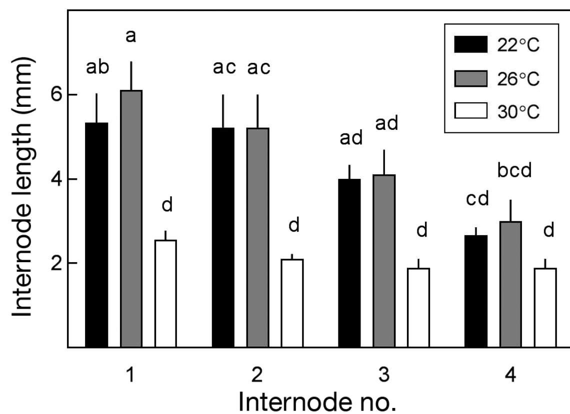

Fig. 4. Length of the 4 youngest rhizome internodes (number 1 is the youngest) of eelgrass shoots grown at 22, 26 and 30°C. Mean values ± SE (N = 3). Different letters indicate significant differences among means (2-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$)

long experiment, while only the first internode at 30°C was in part formed during the experiment. Thus, internodes 2–4 at 30°C reflected the field situation at sampling.

Overall, growth was generally higher in shoots growing at 22 and 26°C, having more leaves as well as wider, longer and visually greener leaves (see Fig. S1 in the Supplement for chl *a*; 1-way ANOVA, not significant) compared to shoots grown at 30°C, which were paler, non-growing and dying. Additionally, roots in shoots at 30°C were rare when shoots were harvested. Thus, most parameters showed much higher leaf and rhizome growth as well as higher shoot production at the 2 cooler temperatures (22 and 26°C) with a clear threshold for decline between 26 and 30°C.

Internal meristematic oxygen

The internal meristematic oxygen content (Fig. S2 in the Supplement) during full day–night cycles showed no systematic differences among shoots grown at different temperatures during daytime or nighttime or between measurements performed during period 1 (Days 6, 7 and 8) or period 2 (Days 20, 21 and 22). During night hours, shoots had relatively high meristematic oxygen content. Minimum oxygen values between 05:00 and 06:00 h, when the oxygen content is expected to be lowest, averaged 37.7 ± 3.9% of air saturation (mean ± SE, N = 17, Table 2). On only 1 occasion was anoxia recorded in a shoot grown at 26°C. During daytime, the oxygen content varied greatly and depended on light levels. Thus, the maximum oxygen values measured at 17:00–18:00 h averaged 123 ± 11% of air saturation. High oxygen content during dark hours and the shoot's ability to elevate meristematic oxygen above 100% during daytime suggest good oxygen supply to the intercalary meristem in all shoots.

TC and TN

Leaf TC content increased with temperature while TN decreased (Fig. 5). TC varied little, but significantly, from 36.9 ± 0.1% (mean ± SE, N = 3) in shoots grown at 22°C to 39.3 ± 0.1% in shoots at 30°C. In contrast, leaf N content declined markedly and significantly to 1.0 ± 0.1% at 30°C compared with 2.7 ± 0.1% at 22°C. The opposing effects of temperature on TC and TN resulted in large differences in C:N atomic ratios from 13.7 ± 0.2 in the 22°C treatment to 39.4 ± 1.9 at 30°C.

Table 2. Minimum and maximum values of dissolved oxygen (% of air saturation) in the intercalary meristem of eelgrass during the first period (Days 6, 7 and 8) and the second period (Days 20, 21 and 22) of the experiment at 22, 26 and 30°C. Minimum values are the lowest values within the time period 05:00–06:00 h, and maximum values are the highest values at 17:00–18:00 h. Mean ± SE, N = 3. See Fig. S2 in the Supplement for more details

Temperature (°C)	Period 1		Period 2	
	Min	Max	Min	Max
22	35 ± 4	114 ± 42	55 ± 5	113 ± 3
26	25 ± 24[a]	157 ± 40[a]	31 ± 12	152 ± 28
30	42 ± 4	104 ± 24	35 ± 8	108 ± 26

[a]N = 2 due to missing value

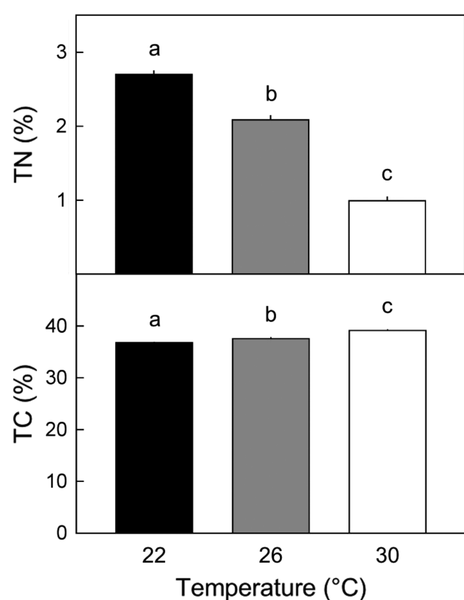

Fig. 5. Total nitrogen content (TN, %) and total carbon content (TC, %) of eelgrass leaves at the end of the experiment for shoots grown at 22, 26, and 30°C. Different letters indicate significant differences among treatments (1-way ANOVA and Tukey's multiple comparison test, $p < 0.05$, N = 3). Mean values ± SE (N = 3)

Metabolomics

Exposure to increasing temperature had substantial impact on the composition of metabolites after the 4 wk treatment (Fig. 6). We annotated 120 compounds of which 59 significantly separated samples among the temperature treatments. There was a significant difference in metabolite compounds in the leaves along the temperature gradient, especially between the 2 lower temperatures (22 and 26°C) and 30°C. Temperature affected N-containing compounds, soluble sugars and metabolites related to reactive oxygen species (ROS). At 30°C, γ-aminobutyric acid (GABA), urea and the amino acids asparagine, glutamine, pyroglutamic acid, aspartic acid, valine, leucine, isoleucine, threonine, proline, serine, glycine, alanine and glutamic acid were decreased compared to 22°C. The content of the soluble sugars, cellobiose, maltose, trehalose and kestose, and also the antioxidants ascorbic acid and α-ocopherol were increased at 30°C. Moreover, α-ketoglutarate, which is a key compound in the tricarboxylic acid (TCA) cycle and GABA shunt, was increased in shoots grown at 30°C. Also, shikimic acid, an important component in the synthesis of the aromatic amino acids, and butyne-1,4-diol, the substrate of the N-containing vitamin B6, accumulated in shoots at 30°C.

DISCUSSION

This study demonstrates that high temperatures can have severe negative effects on eelgrass as leaf growth, leaf formation, rhizome growth and ultimately survival were strongly inhibited at 30°C compared with 26 and 22°C. Our results suggest a threshold for heat stress within the temperature interval of 26–30°C. Our findings were comparable to previous findings showing a 12-fold increase of eelgrass shoot mortality at 25 to 30°C compared to 10 to 20°C in Danish shoots (Nejrup & Pedersen 2008) and field observations of die-backs in Chesapeake Bay in July–August when temperatures reach 30°C (Orth & Moore 1986). In our experiment, shoot density increased at 22°C and declined at 30°C. Accordingly, the zero balance between shoot natality and mortality occurred at a temperature similar to large-scale field observations from Chesapeake Bay showing an inflection point between increase and decrease of eelgrass cover at 23.0°C (see Fig. 2 and Moore et al. 2014). In comparison to shoot demography, the formation of new leaves appeared to be less sensitive to heat stress, as 1 new leaf was formed per shoot every week at both 22 and 26°C. Leaf formation rate in this experiment was higher than previously observed in Chesapeake Bay in late summer (Moore et al. 1996). Such a high leaf turnover may be required, however, to keep pace with shorter leaf longevity at high temperature (Sand-Jensen 1975, Wium-Andersen & Borum 1980) and may have been induced by the suddenly improved conditions following transfer of shoots from higher field temperatures to more moderate experimental temperatures (22 and 26°C). The increased rate of leaf formation led to significantly more leaves on shoots, resembling the response during spring, where improved environmental conditions led to better growth and leaf formation (Moore et al. 1996). Overall, our results, and those of others (Orth & Moore 1986, Moore et al. 2014, Salo & Pedersen 2014, Arnold et al. 2017, Moreno Marín et al. 2018), suggest that warming over relatively short periods and temperature ranges can have very significant negative effects on eelgrass performance at its southern distribution limits and probably also in colder waters experiencing exceptionally warm periods but at lower threshold temperatures compared to our study (Bergmann et al. 2010, Franssen et al. 2011, Gu et al. 2012).

The mechanisms for these temperature effects can be complex and may potentially result in summer

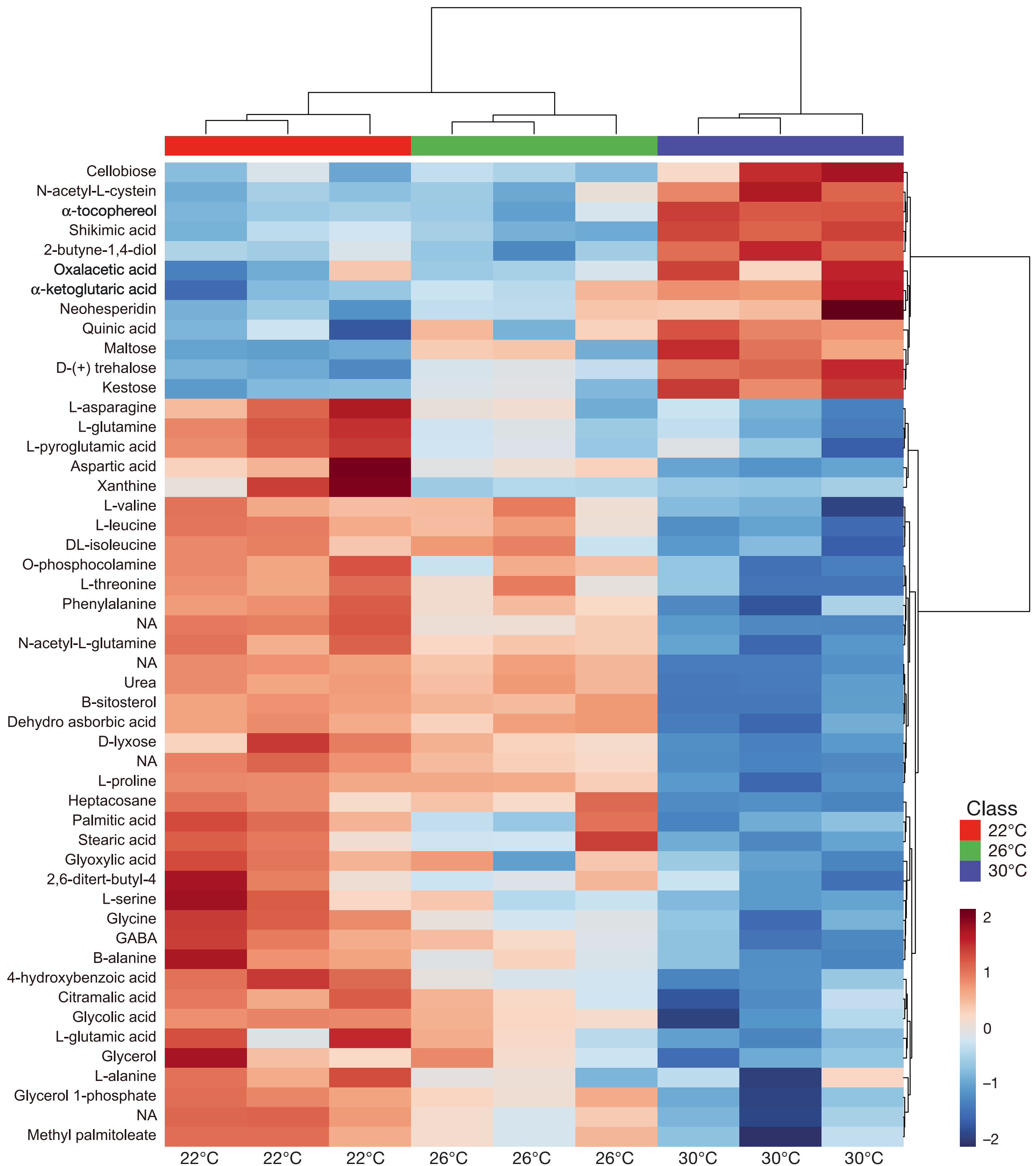

Fig. 6. Heat map showing the relative upregulation (red colors) and downregulation (blue colors) of the 50 metabolic compounds with the most significant changes (1-way ANOVA and Tukey's multiple comparison test, $p < 0.05$, N = 3) in eelgrass leaves growing at 22, 26 and 30°C. Three replicates are shown. NA: not annotated

heat-related eelgrass die-back. We here address 3 principal mechanisms: (1) anoxic stress in intercalary meristem tissue, (2) development of negative whole-plant carbon balance and (3) reprogramming of the metabolic network.

Tissue anoxia

Anoxia in the intercalary meristems has strong negative effects on eelgrass (Pulido & Borum 2010, Raun & Borum 2013), probably due to cell acidifica-

tion, accumulation of toxic metabolites or low energetic outcome from anaerobic metabolism (Crawford & Brändle 1996). At high temperatures, anoxia in the intercalary meristems rapidly causes reduced leaf and rhizome growth, degradation of the meristematic tissue and detachment of shoots from the rhizomes (Greve et al. 2003, Borum et al. 2005). In our experiment, leaf growth was very low, and apparently no new rhizome segments and few new leaves were formed at 30°C. However, we did not observe tissue degradation and detachment of entire green shoots. This is consistent with observations that meristematic tissues remained oxic throughout the diel cycles. The relatively high and constant meristematic oxygen level throughout dark hours were likely supported by continued oxygen diffusion into the shoots from the air-saturated water column (Greve et al. 2003, Borum et al. 2005). Any possible differences in respiration rate among temperature treatments may have been hidden by high variation in oxygen concentration within the meristematic tissue of the individual shoots (Raun & Borum 2013). In addition and as discussed below, root biomass was low at the high temperature treatment reducing the oxygen drain of below-ground tissues.

Shoots exposed to 30°C had no or only few short roots by the termination of the experiment, indicating anoxia and degradation of root tissues. Dying roots among submersed shoots are typically related to anoxia in organic sediments (Raun et al. 2010, Møller & Sand-Jensen 2011). Eelgrass frequently grows in highly reduced sediments and, although well adapted, the apical root meristems buried deep in the sediments are vulnerable to prolonged anoxia due to a high oxygen demand and radial oxygen loss (Jensen et al. 2005). In addition, toxic sulfide is formed in anoxic sediments and may potentially enter roots during dark hours if oxygen supply is insufficient to maintain an oxic micro shield around the root surfaces (Pedersen et al. 2004, Brodersen et al. 2015, Hasler-Sheetal et al. 2016). High temperatures increase oxygen demand due to stimulation of respiration in both shoots and sediment, making roots more prone to anoxia and sulfide invasion (Holmer & Bondgaard 2001). However, it is unclear if the shoots had lost roots during the warm summer period prior to the experiment with subsequent regrowth of roots in shoots incubated at 22 and 26°C, or if shoots incubated at 30°C lost their roots during the experiment. Nevertheless, the loss of roots does not in itself induce shoot mortality, but nonfunctional roots will have major impacts on nutrient uptake and, hence, leaf and shoot growth as discussed below.

Whole-plant carbon balance

A positive whole-plant carbon balance of eelgrass is challenged by high temperatures when respiration rates exceed photosynthesis on a 24 h basis (Marsh et al. 1986). Respiration continuously rises with temperature, while net photosynthesis has an optimum typically between 20 and 30°C (Marsh et al. 1986, Zimmerman et al. 1989, Staehr & Borum 2011, Beca-Carretero et al. 2018). Furthermore, high temperature can reduce the functioning of photosystem II (PSII) (Arbona et al. 2013). The critical temperature for positive eelgrass carbon balance varies among geographical regions as a result of temperature acclimation and adaptation (Zimmerman et al. 1989, Staehr & Borum 2011). However, temperatures ≥30°C are known to result in negative carbon balance in eelgrass (Marsh et al. 1986). To resist longer periods of high temperatures, eelgrass must rely on stored carbon reserves. These nonstructural carbohydrates peak in spring and early summer and drop in late summer as temperature rises and carbohydrate reserves are depleted (Burke et al. 1996). However, in future climate scenarios, increasing CO_2 concentrations may stimulate photosynthesis and thus improve the carbon balance during high temperatures (Zimmerman et al. 2015, 2017). In our experiment, the slow growth and leaf production rates in the heat-stressed shoots at 30°C suggest a negative carbon balance. Nevertheless, we found that most soluble sugars (cellobiose, maltose, trehalose and kestose) accumulated in shoots grown at 30°C compared to shoots grown at 22 and 26°C. This agrees with the finding that sustained photosynthetic activity at all 3 temperatures drove meristematic hypoxia in the dark to supersaturation in light (Fig. S2). In addition, we found that the TC content in the leaves increased with temperature but was within the normal range in eelgrass (Duarte 1990). The high TC and the increase in soluble sugars indicated that the main heat stress factor was not the negative carbon balance or a reduced functioning of PSII. Trehalose, maltose, sucrose and other sugars accumulate in connection with acclimation to heat stress (Gu et al. 2012, Arbona et al. 2013) indicating that shoots were attempting to acclimate to the high temperature. However, sugar accumulation could also result from continued photosynthesis but loss of the ability to synthesize proteins for tissue growth owing to low N uptake or N loss as reflected by both the very low TN content and downregulation of amino acids and other N-containing compounds.

Reprogramming of the metabolic network

We found that the metabolome was markedly altered in shoots grown at 30°C compared to 22 and 26°C. Heat-stressed shoots at 30°C had increased levels of the antioxidants α-tocopherol (vitamin E) and ascorbic acid, indicating a defense against oxidative stress. During heat stress, uncoupling and changes of metabolic processes can lead to an increase of reactive oxygen species (ROS) (Dat et al. 2000, Obata & Fernie 2012), and the accumulation of ROS causes oxidation and damage of cellular compounds and, in the worst case, cell death (Obata & Fernie 2012). Plants have evolved several defensive mechanisms against ROS, where e.g. α-tocopherol exerts a protective effect against heat stress through scavenging of ROS and stabilization of the lipid phase of the thylakoid membranes in the chloroplasts (Arbona et al. 2013).

The N balance of eelgrass shoots was significantly altered when exposed to 30°C. Shoots grown at 22 and 26°C had typical N contents (Duarte 1990), while shoots grown at 30°C contained less than 1% N of dry mass, which cannot be explained by natural variation (Fourqurean et al. 1997). The low N content could potentially be due to impaired N uptake and assimilation because of the degraded roots. N may, however, be taken up by both roots and leaves or translocated from older, non-growing leaves (Short & McRoy 1984, Pedersen & Borum 1992) and, hence, the shoots should have had N sources even with non-functioning roots. Since leaf and root production was close to zero and therefore no new tissues were formed, the results clearly reflect a net loss of N from the temperature-stressed tissues that cannot be explained by impaired N uptake. This net loss instead indicates major damage to tissue integrity, N metabolism and protein/enzyme synthesis. Previous studies have indicated that eelgrass acclimates to heat stress by upregulating genes and increasing metabolites related to cell wall synthesis and strengthening (Franssen et al. 2011, Gu et al. 2012, Jueterbock et al. 2016). However, our results indicate that this mechanism failed at 30°C.

When exposed to 30°C, the high temperature and the net loss of N from shoots correlated with major changes in metabolites, indicating substantial disturbance of metabolic pathways and enzymatic processes. Enzymes have different sensitivity to high temperatures, and while heat might uncouple certain metabolic pathways, others are still functioning, resulting in accumulation of specific metabolites while others are depleted (Mittler et al. 2012). Metabolites associated with N metabolism are often affected during stressful events, although they usually accumulate (Obata & Fernie 2012). GABA, an amino acid and a key metabolite involved in N metabolism (Bouché & Fromm 2004), often accumulates during abiotic stress (Shelp et al. 1999). However, in our experiment, GABA was significantly reduced in shoots grown at 30°C. Likewise, acclimation to heat stress may involve accumulation of amino acids like alanine (Arbona et al. 2013), while we found the opposite response. Previously, increased levels of both GABA and alanine have been found in eelgrass during anoxic stress (Pregnall et al. 1984, Hasler-Sheetal et al. 2015) showing that eelgrass can respond to abiotic stress with increased levels of compounds related to the N cycle. The present opposite response to abiotic stress might be related to the time scale of the experiment. While we measured the metabolic composition after a 4 wk long period of heat stress, other studies investigated short-term (day-to-day) responses to abiotic stress (Pregnall et al. 1984, Hasler-Sheetal et al. 2015). Short-term anoxia stress induces activation of alternative metabolic pathways, while long-term stress and activation of alternative pathways may markedly alter the metabolic pathways and chemical composition in the shoots. In other studies of longer-term (3 to 4 wk) heat stress, genes related to protein degradation were induced in unrecoverable eelgrass shoots (Franssen et al. 2011). The latter result compares with our findings of decreased leaf N, downregulation of N-containing metabolites and the fact that shoots growing at 30°C completely failed to acclimate to the high temperature.

We found additional indications of disruption of N metabolism within leaves at 30°C. Aromatic amino acids are synthesized via the shikimate pathway, where shikimate is a precursor in the pathway leading to amination of amino acids (Herrmann 1995). While we found depletion of the aromatic amino acids phenylalanine, tyrosine and tryptophan in shoots grown at 30°C, shikimate was upregulated. This accumulation indicates disruption further along the synthesis pathway of amino acids, possibly at the amination process, causing shikimic acid to accumulate. Also, the precursor of the N-containing vitamin B6, butyne-1,4-diol, accumulated as an additional indication of disruption of the amination process. Inhibition of the amination process may have been caused by enzyme inhibition or simply the shortage of inorganic N. For future research on temperature effects on metabolic changes in eelgrass, additional analyses of genes (reverse transcription-qPCR) along with analyses of antioxidants and sugar metabolism would provide a further, in-depth knowledge on metabolomic changes induced by heat stress.

CONCLUSION

The aim of this study was to investigate temperature effects on eelgrass growth and survival close to its southern distribution limit in Chesapeake Bay and to examine possible physiological explanations for negative temperature effects. As hypothesized, heat stress reduced growth and shoot density. The results demonstrate a threshold for temperature stress in eelgrass at its southern distribution limit in the range between 26 and 30°C, as shoots completely failed to acclimate to 30°C. We also hypothesized that increasing temperatures would lead to lower meristematic oxygen concentration in the dark due to increased respiration, but we did not find support for this. The continuously high oxygen content in all measured shoots indicates that the observed negative effects were caused directly by temperature rather than by anoxia in the intercalary meristem, although root degradation observed at 30°C could likely have been induced by oxygen shortage in these distal tissues. High temperature had major impacts at the cellular level by substantially reprogramming the metabolic network. High temperature increased levels of antioxidants and substantially reduced TN, amino acids and other compounds related to the N cycle. We interpret this response as being a failed attempt to adapt to high temperature and being due to impairment of the shoot's ability to maintain N balance and metabolic homeostasis.

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