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ORIGINAL PAPER

Genotypic variation influences tolerance to warming and acidification of early life-stage *Fucus vesiculosus* L. (Phaeophyceae) in a seasonally fluctuating environment

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Abstract Global change exposes brown algal *Fucus vesiculosus* populations to increasing temperature and pCO_2, which may threaten individuals, in particular the early life-stages. Genetic diversity of *F. vesiculosus* populations is low in the Baltic compared to Atlantic populations. This might jeopardise their potential for adaptation to environmental changes. Here, we report on the responses of early life-stage *F. vesiculosus* to warming and acidification in a near-natural scenario maintaining natural and seasonal variation (spring 2013–2014) of the Kiel Fjord in the Baltic Sea, Germany (54°27′N, 10°11′W). We assessed how stress sensitivity differed among sibling groups and how genetic diversity of germling populations affected their stress tolerance. Warming increased growth rates of *Fucus* germlings in spring and in early summer, but led to higher photoinhibition in spring and decreased their survival in late summer. Acidification increased germlings' growth in summer but otherwise showed much weaker effects than warming. During the colder seasons (autumn and winter), growth was slow while survival was high compared to spring and summer, all at ambient temperatures. A pronounced variation in stress response among genetically different sibling groups (full-sib families) suggests a genotypic basis for this variation and thus a potential for adaptation for *F. vesiculosus* populations to future conditions. Corroborating this, survival in response to warming in populations with higher diversity was better than the mean survival of single sibling groups. We conclude that impacts on early life-stages depend on the combination of stressors and season and that genetic variation is crucial for the tolerance to global change stress.

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Introduction

Anthropogenic global change exposes marine populations, inter alia, to increases in temperatures and pCO_2 concentrations (IPCC 2013). The magnitude of these changes varies among geographic regions; in the Baltic Sea, warming and acidification are expected to increase up to 3–6 °C and to 1000 µatm, respectively, by 2100 (Graham et al. 2008; Elken et al. 2015). In many regions of the Baltic Sea, *F. vesiculosus* represents the dominant perennial large brown alga (Wahl et al. 2015a). On Baltic intertidal and shallow subtidal shores, its ecological role is important for both the biotic and the abiotic environment. As a foundation species, *F. vesiculosus* provides a three-dimensional habitat for a large number of epibionts and other associated organisms. It plays a key role in community structuring and provides shelter and food to the invertebrate community (Lotze et al. 2001; Wikström and Kautsky 2007). Rockweeds also provide various important ecosystem services including exceptionally high carbon retention (Schmidt et al. 2011) and buffering of environmental changes: the fixation of

carbon and uptake of nitrogen provides the associated community, at least temporally, with favourable conditions regarding nutrients, oxygen and pH (Bertness and Leonard 1997; Wahl et al. 2015c). Therefore, intertidal and subtidal *F. vesiculosus* may, to some extent, buffer physical stress for many other community members, such as primary consumers (herbivores), secondary consumers (carnivores) and other associated organisms (epiphytes).

In the twentieth century, a drastic decline has led to great losses of *F. vesiculosus* biomass, as, e.g., by almost 95 % in Kiel Bay, Western Baltic Sea (Vogt and Schramm 1991), and surveys covering large parts of the Baltic Sea report marked disappearance of *F. vesiculosus* from deeper zones (Eriksson et al. 1998; Torn et al. 2006). Abiotic factors such as eutrophication and decreased irradiation have been proposed to be the most important drivers for this decline (Berger et al. 2004). However, declines of the Baltic bladderwrack are rather due to multiple factors (Wahl et al. 2011), where indirect effects may outweigh direct effects (Wahl et al. 2015a). For instance, heavier nutrient loads lead to higher phytoplankton densities and enhanced growth of epiphytes with the former reducing light penetration and the latter shading the macroalgae thallus (Rohde et al. 2008).

The increase in water temperature and pCO_2 concentrations may further impact *F. vesiculosus* populations by direct and indirect effects and possibly cause distributional shifts or range contractions. It was demonstrated that high-pCO_2 conditions increased photosynthetic rates of *F. vesiculosus* and enhanced growth in *F. vesiculosus*, *F. serratus* and other non-calcifying macroalgae (Gordillo et al. 2001; Nygård and Dring 2008; Olischläger et al. 2012, 2013). The assumed explanation for a positive CO_2 effect is the down-regulation of energy-consuming carbon-concentrating mechanisms (CCMs) under these conditions (Beardall and Giordano 2002; Wu et al. 2008). In contrast, warming causes negative effects on the physiological performance of *F. vesiculosus* populations (Graiff et al. 2015), particularly in the warm season. Thus, a poleward shift of seaweed populations has been observed since 1940 until present as a response to increased warming (Wernberg et al. 2011) and is predicted to continue for *F. vesiculosus* populations in the twenty-first and twenty-second century (Jueterbock et al. 2013). As a consequence of warming, further range retractions and local extinctions of *F. vesiculosus* populations may occur (Nicastro et al. 2013). To date, the combined effects of CO_2 and temperature increase on *F. vesiculosus* performance have not been investigated yet.

Early life-stages of seaweeds may be particularly sensitive towards global change stress and environmental fluctuations (Coelho et al. 2000). Post-settlement mortality of young *F. vesiculosus* is generally high in the field, but varies among seasons and years (Lamote and Johnson 2008). Similarly, elevated temperature impacts growth rates, survival, photosynthetic efficiency and sensitivity to copper more strongly in *F. serratus* germlings than in adults (Nielsen et al. 2014). A decreased germination success of Baltic *F. vesiculosus* at 25 °C compared to 15 °C was observed by Maczassek (2014). Since this early ontogenetic stage may constitute the bottleneck of macrophyte survival, it is crucial to assess how a combination of expected future temperature and pCO_2 will influence the performance of *F. vesiculosus* germlings. It is also unknown to which extent variations in responses to these two factors have a genetic basis in *F. vesiculosus*. Since selection is only effective if variation on traits responsive to global change factors exists, this knowledge is important to assess the adaptive potential of a species (Caruso et al. 2005).

Population resistance to global change stress is mediated by a high genetic diversity which guarantees a broad adaptive potential (Frankham et al. 2009). The loss of genetic diversity restricts the potential for adaptation (Frankham 2003, 2010; Hoffmann and Sgro 2011; Pauls et al. 2013). Genetic diversity of the eelgrass *Zostera marina* enhanced ecosystem resilience under a summer heat wave (Reusch et al. 2005; Ehlers et al. 2008) as well as community resistance to grazing by geese (Hughes and Stachowicz 2004). Also at early life-stages, genetic diversity improved recruitment of the barnacle *Balanus improvisus* (Gamfeldt et al. 2005) and enhanced the early life-stage performance in the solitary ascidian *Ciona intestinalis* (Aguirre and Marshall 2012).

Adaptive responses to climate change occur more on the population than on the species level (Harvey et al. 2014) and may be particularly critical in the Baltic Sea, where species and genetic richness is lower compared to other ecosystems (Pereyra et al. 2009). The Baltic Sea, a semi-enclosed water basin formed 8–10 thousand years ago, constitutes a geographically and ecologically marginal ecosystem with particular environmental conditions due to a gradient of decreasing salinity towards from the North Sea (32 psu) towards the north-eastern part (Bothnian Bay <4psu) (Bonsdorff 2006). This steep salinity gradient and restricted water exchange at the entrance of the Baltic Sea contribute to the lower genetic diversity of Baltic *F. vesiculosus* populations *versus* North Sea populations and to high genetic distances between them (Johannesson and André 2006; Tatarenkov et al. 2007). These remarkable differences may have additionally derived from low population connectivity due to limited egg dispersal in *F. vesiculosus* (Serrão et al. 1996). Despite its ecological importance, our understanding about adaptive processes in *F. vesiculosus* under global change conditions are still scarce and require further investigations (Johannesson et al. 2011).

In the present study, the single and combined effects of warming and acidification on *F. vesiculosus* germlings were

assessed in all four seasons in genetically different sibling groups (families) and in populations of differing genetic diversity. Experimental populations of *F. vesiculosus* in different artificially generated diversity levels (low, medium and high) were treated with current and future temperature and pCO_2 conditions to explore whether diversity effects exist. We hypothesised that (1) the responses of *F. vesiculosus* to single and combined effects to temperature and pCO_2 vary with season, (2) genetically dissimilar germling groups (full-sib families of germlings) differ among each other in their response to single and combined effects of temperature and pCO_2 and (3) genetically more diverse experimental groups of *F. vesiculosus* are more resilient to stress than genetically less diverse groups.

Materials and methods

Experimental approach

To test the existence of sensitivity differences among genetically dissimilar sibling groups (full-sib families of germlings), sibling groups were produced following a controlled protocol. Since all germlings within one sibling group had the same mother and father, they were all 'sisters and brothers', i.e. full-sibs. Other sibling groups had other parents. We thus assume that genetic variability within families was substantially less than that among families. These eight genetically different families were placed in each of 12 mesocosms (termed Kiel Outdoor Benthocosms below). This 'common garden'-setting reducing between-family environmental differences to a minimum allows us to assume that between-family differences in responses has a strong genetic basis. To test hypothesis (2), we used sibling group as fixed factor.

To test hypothesis (3), the differences in responses between diversity levels, we produced three artificial diversity levels: one-family experimental populations, where all germlings came from the same parental pair (low diversity level); two-family mixtures, where equal numbers of germlings were taken from two families (medium diversity level) and four-family mixtures, where equal numbers of germlings were taken from four families (high diversity level). Hence, in the two- and four-family mixtures, germlings from any one family could account for no more than 50 and 25 % of the experimental population, respectively.

Sampling, gamete acquisition, rearing of germlings and experimental design

We experimentally studied germlings' performance in all four seasons. In the German Baltic Sea, individuals of *F. vesiculosus* reproduce in summer or autumn or even in both seasons (Maczassek 2014). *F. vesiculosus* germlings were allowed to settle on sandstone cubes with 2 cm edge length, where each sandstone represents an experimental population. To cover all four seasons of the year, two cohorts of germlings were used to establish the experimental populations. 'Cohort 1' originated from autumn 2012 spawning *F. vesiculosus* and was used in the experiments from January 2013 to September 2013, and 'cohort 2' originated from summer 2013 spawning *F. vesiculosus* and was exposed to the experiments from September 2013 to March 2014.

For producing cohort 1, a total of 53 fertile specimens (33 females, 20 males) of *F. vesiculosus* were collected in a wave-exposed area with mainly hard substrate located in the south-western Baltic Sea (Bülk, Germany, 54°27.327′N, 10°11.977′W) during the end of November 2012. Individuals collected were distanced above two metres which is the estimated maximum dispersal distance of most *F. vesiculosus* eggs (Serrão et al. 1996), thereby reducing the probability of sampling of siblings and enhancing genetic variability. Algae were immediately transported to the laboratory after collection in cooler boxes. Fertile receptacles were cut from male and female algae, rinsed with tap water, blotted dry and stored in the dark at 8–10 °C for 5 days. To generate full-sib germling groups, all receptacles of one female and all receptacles of one male were each combined in plastic dishes by random pairing. Gamete release (and the following fertilisation) was induced by the immersion of the receptacles in sand-filtered seawater (15–16 psu) and exposing them to light irradiated from an aquarium lamp (110 µmol photons m^{-2} s^{-1}) for 3 h. The term 'sibling group' is used hereafter to represent an experimental population, where all germlings were full-sibs settled on a sandstone surface. For cohort 1, eight sibling groups were generated, which were genetically different among each other, since they stemmed from different parents. Germlings were settled as follows: a 0.67-ml solution of fertilised eggs from one parental pair was pipetted homogeneously onto a 2 × 2 cm sandstone surface. Hence, single sandstones bore descendants from one single parental pair. From cohort 1, eight different sibling groups were established and replicated three times (methodological replicates of exactly the same offspring identities in each of the separate benthocosm tanks). Germlings were cultured and their presence on each sandstone was monitored weekly for 8 weeks in a room with windows allowing for natural light conditions at 8 °C with weekly water exchange (15–16 psu) until transfer to the Kiel Outdoor Benthocosms (see 'Experimental conditions: Kiel Benthocosms' section).

Cohort 2 was produced with the same method regarding sampling and gamete acquisition, but here a total of 66 fertile specimens (36 females, 30 males) were collected in June 2013. Receptacles were stored in the dark at 15 °C for 8 days prior to introducing gamete release and fertilisation.

For cohort 2, different levels of genetic diversity were generated. The term 'diversity level' is used hereafter to denote different compositions of experimental germling populations settled on the sandstone surface: the low diversity level, consisting of single sibling groups, was generated by pipetting a 0.75-ml solution of fertilised eggs from one parental pair each onto each sandstone surface. To generate the medium diversity level, a 0.375-ml solution of fertilised eggs from each of two parental pairs (pairs) was combined onto each sandstone surface. To generate the high diversity level, a 0.1875-ml solution of fertilised eggs from each of four parental pairs was combined onto each sandstone surface (quartets). In total, a 0.75-ml solution of fertilised eggs was pipetted homogeneously onto every sandstone cube. Germlings were cultured and monitored over the following 9 weeks at 15 °C until transfer to the Kiel Benthocosms. Eight low diversity level experimental populations, four medium diversity level populations and two high diversity level populations were generated, with each population being produced by different parents: i.e. parent identities were replicated. This whole set-up was replicated (methodological replicates of exactly the same offspring identities in each of the separate benthocosm tanks). For an illustration of the experimental design, see Online Resource 1. Germlings were cultured over the following 9 weeks at 15 °C until transfer to the Kiel Benthocosms.

Experimental conditions: Kiel Outdoor Benthocosms

The experiments were performed at the near-natural scenario in the Kiel Outdoor Benthocosms (KOB) under natural ambient and manipulated temperature and CO_2 conditions at 2 levels ('present' and '2110'). 'Present' conditions were the actual environmental conditions in Kiel Fjord transferred to the experimental tanks via continuous flow-through (1 tank-volume per day, i.e. 1500 l/24 h). '2110' conditions were simulated by adding 5 °C to the actual fjord temperature, as it has been predicted for the next 100 years (Elken et al. 2015). pCO_2 was increased in the hooded headspace above the tanks to 1100 µatm, according to the predictions by Schneider et al. (2015). These two delta-treatments increased the mean of the respective factors while preserving the frequency and amplitude of natural fluctuations. During all seasons, abiotic factors were logged continuously, nutrients and total alkalinity (TA) were measured twice a week and dissolved inorganic carbon (DIC) was measured monthly. The technical set-up and all measured parameters are described in detail by Wahl et al. (2015b). In addition, temperature was measured daily with a calibrated sensor (pH, Mettler Toledo GmbH, Giessen, Germany) (Online Resource 2) and the seasonal variation in solar radiation was measured in the Kiel Fjord by Rickert et al. (2015) (Online Resource 3). Light intensities (µmol photons m^{-2} s^{-1}) of the Kiel Fjord were reduced by ca. 20 % in the Kiel Benthocosm tanks, as shown by Wahl et al. (2015b). Across the 12 experimental units of KOB, the two factors warming and acidification were orthogonally crossed, creating the four treatment levels: T+CO_2+ (warmer and acidified), T+CO_2− (warmer, non-acidified), T−CO_2+ (ambient temperature, acidified) and T−CO_2− (ambient temperature, non-acidified) in three replicates. 'Non-acidified' refers to the ambient CO_2 conditions of Kiel Fjord with naturally fluctuating, sometimes acidified conditions, but without receiving an additional pCO_2 treatment in the KOB. All experimental populations (sibling groups of cohort 1 and 2 and diversity level of cohort 2) were introduced to each of the experimental units of KOB and were hence exposed to each of the four treatment levels with three replicates per treatment. Within the Bioacid II project and the 'Benthic Consortium', experiments of different disciplines were performed (www.bioacid.de). In each tank, a benthic community was analysed in the KOB consisting of macrophytes (adult *F. vesiculosus*) and their associated epibiotic communities as fauna, flora and consumers. These experiments were subdivided into four consecutive experiments (E1, E2, E3 and E4) each run for 11–12 weeks, covering all four seasons of the year: E1: spring (01.04.2013–21.06.2013), E2: summer (01.07.2013–20.09.2013), E3: autumn (07.10.2013–20.12.2013) and E4: winter (13.01.2014–01.04.2014).

Prior to the first experiment with cohort 1, germlings were acclimatised to the treatment conditions for 9 weeks. Temperature was increased gradually over 2 days until reaching the +5 °C treatment. When tanks were serviced in between experiments, germlings were stored in indoor mesocosms at same temperature and pCO_2 conditions as in the benthocosms for 1–3 weeks. Germlings were kept in four indoor mesocosms, one for each treatment. The temperature treatment was established by using internal heater elements (600 W, Schego Titan, Schemel & Goetz, Offenbach am Main, Germany), and values were set according to the actual fjord temperatures for the ambient treatment and +5 °C for the warmed treatment. Acidification was achieved by aeration with CO_2-enriched air (1000 µatm CO_2) directly into the water of the mesocosms. pH and temperature of the mesocosms were monitored daily. Between the core experiments E2 and E3 (cohort 2), technical problems in one of the indoor mesocosms damaged germlings of the treatment T−CO_2+ and led to the deletion of this treatment combination. To compensate this loss, a new experiment was initiated. Twenty-one sibling groups previously treated at ambient conditions as supplementary material (with three replicates) were distributed randomly (illustrated by a non-significant ANOSIM among the created communities, p value >0.05) to the four treatment levels described above resulting in five sibling groups in

each benthocosm tank. The new experiment covered all four treatment levels and was used for survival and growth analysis for the following seasons. The previous experiment, consisting of the three treatment levels ($T+CO_2+$, $T+CO_2-$ and $T-CO_2-$), was used for diversity level and sibling group difference analysis.

From summer until the end of the experiments, sibling groups had to be protected from germlings originating from fertile adult *F. vesiculosus* kept in the same tanks. Therefore, germlings were kept in PVC boxes (70 cm × 40 cm × 12 cm). These boxes were enclosed to the water content of the tank but uncovered towards the upper part and positioned above the water surface of the benthocosm tank while separating the water contents from each other. The thin PVC walls (6 mm) allowed for temperature exchange and the open upper part allowed for gas exchange. The box water was exchanged once a week by filtered water (50 µm) of the benthocosms. The filtration prevented the accidental introduction of new *F. vesiculosus* eggs (100 µm diameter), but exchanged water with the temperature and gas conditions of the benthocosm tanks. The light intensity (µmol photons m^{-2} s^{-1}) in the box was 5 % lower than in the main tank.

Survival

Germlings' survival was determined as the % of surviving germlings between the beginning (t_0) and the end (t) of each time period. All germlings on a 1-cm^2 area were counted under a binocular at 25× magnification. When a given cohort was used in two successive time periods, the number of survivors (t) of the previous experiment was taken as the start number for the following season. Survival was calculated for all time periods of the year. Summer was separated into the two time periods early summer and late summer; thus, temperatures of the warming treatment reached values above the tolerance range (>27 °C) of *F. vesiculosus* (Graiff et al. 2015). Survival % for spring, early summer, late summer, autumn, winter 2013 and for spring 2014 were calculated as:

$$\text{Survival } \% = \frac{\text{Number } t}{\text{Number } t_0} \cdot 100$$

Growth

Digital images were recorded of 10–15 individual germlings per sibling group at 40× magnification (SteREO Discovery. V8—Carl Zeiss Jena GmbH), according to Steen and Scrosati (2004). The projected side-view area of individual germlings was measured using the image analysis software Image J 1.45s (National Institutes of Health, USA). For each of the four treatments, eight sibling groups were measured for cohort 1 and five sibling groups for cohort 2 at the beginning and end of a time period of 6–9 weeks. These time periods were chosen for achieving uniform analysis over the year. In summer, growth was measured separately in early summer and late summer. Hence, time periods for growth measurements were in spring, early summer, late summer, autumn, winter 2013 and for spring 2014.

Relative growth rate (RGR) in % d^{-1} was calculated according to the exponential growth rate as

$$\text{RGR} = \left[(\text{Area } t/\text{Area } t_0)^{1/\Delta t} - 1\right] \cdot 100$$

where Area t_0 is the mean area of eight sibling groups (five sibling groups for cohort 2) at day 0, Area t is the mean area at day t and Δt the number of days between t and t_0. From each experimental population, the mean of 15 individuals in Area t and Area t_0 was calculated. Since individuals were too small for labelling, the 15 individuals were chosen randomly for each measurement. In successive experiments, Area t, the area measured at the end of the previous experiment, was set as the initial size Area t_0 of the subsequent experiment. Negative growth rates were given when bigger individuals within a sibling group died while smaller ones remained.

Chlorophyll a fluorescence parameters

In order to determine the photosynthetic parameters of *F. vesiculosus* germlings, the in vivo chlorophyll *a* fluorescence of photosystem II (PSII) was measured with a portable pulse-amplitude-modulated fluorometer (Pocket PAM, Gademann Instruments GmbH, Würzburg, Germany) at the end of the experiments (March 2014). Before each measurement, the germlings were carefully cleaned of epiphytes with a brush and seawater. The distance between the fibre optic (diameter 1.5 mm) and the germling was always kept constant at 1 mm with the help of a binocular and a micromanipulator. The determination of the potential maximum quantum yield (F_v/F_m) was performed according to Hanelt (1998). After 5 min of dark adaptation and a 5 s of far red light, the minimal fluorescence F_0 was recorded with a pulsed measuring light (650 nm, 0.3 µmol photons m^{-2} s^{-1}), followed by short pulses of completely saturating white light pulse (0.4–0.8 s, 1000–5000 µmol photons m^{-2} s^{-1}) to record F_m ($F_v = F_m - F_0$). The relative PSII electron transport rate (rETR) of each germling was quantified by incubations for 5 min in darkness and followed by exposures to nine increasing photon flux densities (PFD) of actinic red light (0, 25, 45, 66, 90, 125, 190, 285 and 420 µmol photons m^{-2} s^{-1}, LED 650 nm), each for 1 min. The photosynthesis vs. irradiance curves (PI curves) with rETR as a function of PFD were fitted after Walsby (1997). From each single curve, the maximum relative electron

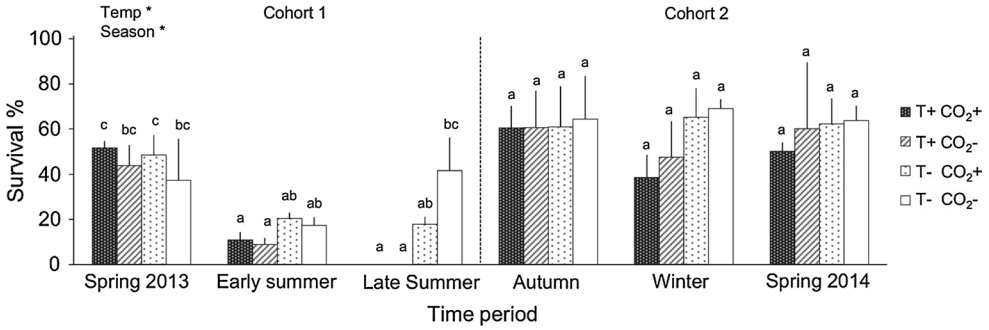

Fig. 1 Germlings' survival (%, mean +SD, $n = 3$, in 7–8 weeks) at the four treatment combinations: T+CO_2+, T+CO_2−, T−CO_2+ and T−CO_2− over a period of the year 2013 and spring 2014. Means were calculated across eight sibling groups (cohort 1) and across five sibling groups (cohort 2) within one benthocosm tank. Significant effects are indicated here (*asterisk*) after the repeated-measures ANOVA for cohort 1 (from spring 2013 through early summer and late summer) and cohort 2 (from autumn 2013 through winter and spring 2014). In cohort 2, the temperature effect was insignificant ($p = 0.0556$). Different letters above the bars indicate significant differences (p value <0.05) between the treatments after Tukey's test

transport rate ($rETR_{max}$) and the light saturation coefficient of the curves (I_k) were calculated. In addition, the non-photochemical quenching (NPQ) for each PFD was recorded and calculated (Govindjee 1995). For a quantitative description of NPQ as a function of PFD, NPQ versus PFD curves were fitted after Serôdio and Lavaud (2011). Depending on this model, NPQ_{max} as the maximum NPQ value of the curve was calculated.

Variance analysis

The variance between groups of each diversity level was analysed to explore variations between groups of the low diversity level relative to the variance between experimental populations of the high diversity level. For the low diversity level, the variances between sibling groups were calculated; for the medium diversity level, the variance between pairs and for the high diversity level, variances between quartets were calculated. The cohort 2 was chosen for the variance analysis in order to compare three diversity levels. Variances were calculated as: Variance $= \frac{\sum (x-\tilde{x})^2}{n}$

Statistical analysis

Growth rates (% d^{-1}) and survival (%) values were analysed in a repeated-measures ANOVA for each cohort, where temperature, CO_2 and time period were fixed factors and sibling group a random factor. Survival % values were arcsin-transformed. The factor time period defined the repeated measures. Due to enhanced mortality in the high-temperature treatments during late summer, for this period only the factor CO_2 was analysed in a mixed-model ANOVA with the fixed factor CO_2 and the random factor sibling group. Diversity level differences were analysed for cohort 2 in a mixed-model ANOVA with regard to the response variables growth rate (% d^{-1}) and survival (%): the fixed factors were treatment (three treatment levels: T+CO_2+, T+CO_2− and T−CO_2−) and diversity level (three treatment levels: low, medium and high), while sibling groups were treated as a random factor. Differences between sibling groups were tested with a three-way ANOVA with the factors temperature, CO_2 and sibling group for each time period with regard to survival (arcsin-transformed) and relative growth rate.

For the chlorophyll *a* fluorescence parameter, a mixed-model ANOVA was used to test the two fixed factors temperature and CO_2 (two levels: high and low) with the random factor sibling group. Data were tested for normality and for homogeneity of variances. The ANOSIM analysis was performed with Primer 6 (Clarke 1993), and all other analysis, including model assumptions for ANOVA, were performed using R (R Development Core Team 2014).

Results

Survival

Germlings' survival over the year ranged between 0 ± 0 % in late summer and 69.09 ± 4.18 %, $n = 3$ in winter (Fig. 1) and differed significantly among the time periods until late summer [time period, $F(2,253) = 41.08$, $p < 0.0001$; Table 1]. The high-temperature treatment reduced survival significantly to ca. 50 and 0 % in summer and late summer, respectively [temperature, $F(1,253) = 14.45$, $p < 0.001$; Table 1]. While increased temperature had no effect on survival in spring and autumn, survival decreased in winter from 69.09 ± 4.18 to 47.37 ± 16.07 %, $n = 3$ under non-acidified conditions and from 65.22 ± 12.89 to 38.65 ± 9.99 % under acidified conditions. Although

Table 1 Results of the repeated-measures ANOVA testing the effect of the fixed factors temperature, CO_2 and time period on survival (%) including the random factor sibling group for (a) cohort 1 (spring, early summer and late summer) and (b) cohort 2 (autumn, winter and spring 2014)

Source of variation	numDF	denDF	*F* value	*p* value
(a) Cohort 1				
Temperature	1	253	14.451	0.001
CO_2	1	253	0.001	0.981
Time period	2	253	41.075	<0.001
Temp × CO_2	1	253	1.209	0.273
Temp × time period	2	253	7.643	<0.001
CO_2 × time period	2	253	3.073	0.048
Temp × CO_2 × time period	2	253	1.410	0.246
(b) Cohort 2				
Temperature	1	148	3.721	0.056
CO_2	1	148	0.587	0.445
Time period	2	148	0.801	0.451
Temp × CO_2	1	148	0.239	0.625
Temp × time period	2	148	1.046	0.354
CO_2 × time period	2	148	0.726	0.486
Temp × CO_2 × time period	2	148	0.242	0.785

the effect was not significant, due to the low *p* value we consider it likely that a temperature effect existed and would be significant at higher replication [temperature, $F(1,148) = 3.72$, $p = 0.056$; Table 1]. Acidified conditions tended to increase survival in spring 2013 from 37.33 ± 18.37 to 48 ± 8.88 % at ambient and 43.71 ± 9.1 to 51.56 ± 3.09 % at high-temperature conditions, decreased survival in late summer from 41.56 ± 14.67 to 17.82 ± 3.33 % at ambient temperatures although not significantly [CO_2, $F(1,253) = 0.0006$, $p = 0.981$; Table 1], whereas there was no CO_2 effect on survival during the other time periods. The importance of time periods is reflected in the significant interaction between CO_2 and time period [CO_2 × season, $F(2,253) = 3.07$, $p = 0.048$; Table 1].

Growth

Germlings' relative growth rate ranged from -0.14 ± 0.19 % d^{-1} in winter to 4.95 ± 0.43 % d^{-1}, $n = 3$, in late summer under ambient temperature and acidified conditions (Fig. 2). In summer, the high-temperature treatment increased growth significantly from 0.52 ± 0.46 to 1.67 ± 0.33 % d^{-1} under acidified and from 0.04 ± 0.93 to 2.63 ± 1.45 % d^{-1} under non-acidified conditions [temperature, $F(1,134) = 8.19$, $p = 0.005$; Table 2]. High temperatures affected growth stronger in summer than in spring, as underscored by the significant interaction between temperature and time period [temperature × time period, $F(1,134) = 4.79$, $p = 0.03$; Table 2]. Acidification had a tendency to increase growth during late summer from 3.45 ± 1.03 to 4.95 ± 0.43 % d^{-1} at ambient temperatures, though not significantly [CO_2, $F(1,7) = 0.925$, $p = 0.3682$; Table 2]. In cohort 1, growth varied under ambient conditions between 3.33 ± 0.45 % d^{-1} in spring, 0.04 ± 0.93 % d^{-1} in summer and 3.45 ± 1.03 % d^{-1} in late summer [time period, $F(1,134) = 22.82$, $p < 0.001$; Table 2]. Variations among time periods at ambient conditions were also significant in the cohort 2, where growth was 2.91 ± 0.94 % d^{-1} in autumn but decreased considerably in winter with 0.23 ± 0.28 % d^{-1} and in spring 2014 with 0.63 ± 0.18 % d^{-1} under ambient conditions [time period, $F(2,105) = 67$, $p < 0.001$; Table 2].

Growth rates in all treatment levels from November 2013 until March 2014 did not exceed 0.63 ± 0.18 % d^{-1}

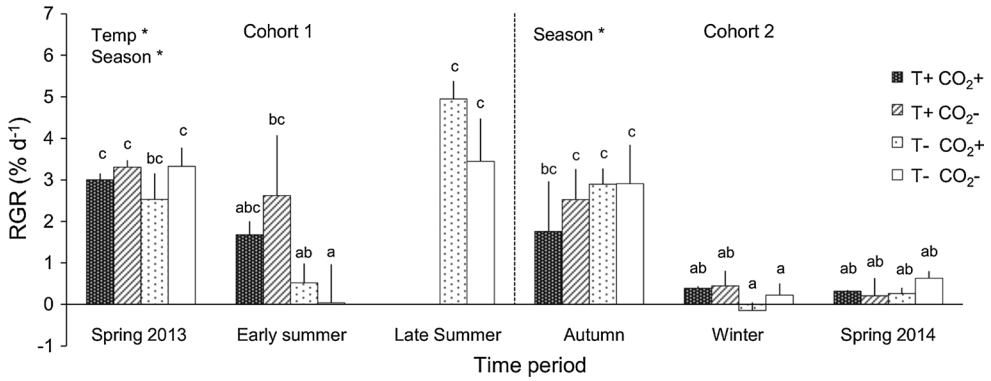

Fig. 2 Germlings' relative growth rate (% d^{-1}, mean + SD, $n = 3$, in 6–8 weeks) at the four treatments combinations: T+CO_2+, T+CO_2−, T−CO_2+ and T−CO_2− over a period of the year 2013 and spring 2014. Means were calculated across eight sibling groups (cohort 1) and across five sibling groups (cohort 2) within one benthocosm tank. Significant effects are indicated here (*asterisk*) after the repeated-measures ANOVA for cohort 1 (from spring 2013 through summer) and cohort 2 (from autumn, through winter and spring 2014). Different letters above the bars indicate significant differences (*p* value <0.05) between the treatments after Tukey's test

Table 2 Results of the repeated-measures ANOVA testing the effect of the fixed factors temperature, CO_2 and time period on the relative growth rate (% d^{-1}) including the random factor sibling group. Cohort 1 (a) include the spring and early summer and cohort 2 (c) include the autumn, winter and spring 2014. Results of the mixed-model ANOVA (b) testing the effect of the fixed factor CO_2 and on the relative growth rate (% d^{-1}) including the random factor sibling group

Source of variation	numDF	denDF	*F* value	*p* value
(a) Cohort 1 (spring–early summer)				
Temperature	1	134	8.194	0.005
CO_2	1	134	1.734	0.190
Time period	1	134	22.824	<0.001
Temp × CO_2	1	134	0.363	0.548
Temp × time period	1	134	4.788	0.03
CO_2 × time period	1	134	0.027	0.87
Temp × CO_2 × time period	1	134	1.578	0.211
(b) Cohort 1 (late summer)				
CO_2	1	7	0.925	0.368
(c) Cohort 2 (autumn–spring 2014)				
Temperature	1	105	0.520	0.472
CO_2	1	105	1.951	0.166
Time period	2	105	67.004	<0.001
Temp × CO_2	1	105	0.737	0.393
Temp × time period	2	105	2.923	0.058
CO_2 × time period	2	105	0.069	0.933
Temp × CO_2 × time period	2	105	1.009	0.368

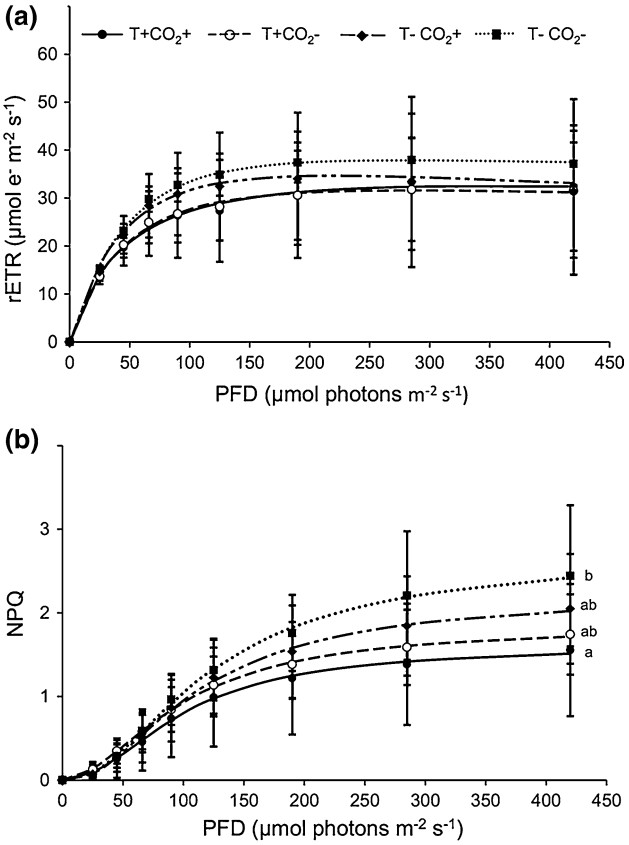

Fig. 3 Effect of the four treatments combinations: T+CO_2+, T+CO_2−, T−CO_2+ and T−CO_2− on photosynthesis irradiance curves **a** mean relative electron transport rate (rETR) and **b** non-photochemical quenching (NPQ) as a function of increasing photon flux density (PFD) of *Fucus vesiculosus* germlings measured at the end of March 2014. Data expressed as the mean ± SD ($n = 3$). Different letters indicate significant differences (p value <0.05) between the treatments after Tukey's test

and were lower compared to growth rates in autumn. While in October the high-temperature treatment reached 19 °C, values fell below 13 °C in November and 8 °C in January (Online Resource 2).

Chlorophyll a fluorescence parameters

The maximum quantum yield (F_v/F_m) did not differ significantly among the treatments. Mean values for F_v/F_m (±SD) for T+CO_2+, T+CO_2−, T−CO_2+ and T−CO_2− were 0.62 (±0.06), 0.65 (±0.05), 0.65 (±0.05) and 0.67 (±0.04), respectively. The maximum electron transport rate ($rETR_{max}$) of *F. vesiculosus* germlings measured in March 2014 tended to be slightly higher under ambient (38.5 µmol e^- m^{-2} s^{-1}) as compared to warmer and acidified conditions (31.9 µmol e^- m^{-2} s^{-1}) (Fig. 3a). Accordingly, the light saturation point I_k had a mean of 45.8 ± 16.2 µmol photons m^{-2} s^{-1} under ambient conditions, whereas at acidified conditions I_k reached mean values with 41.7 ± 18 µmol photons m^{-2} s^{-1}. However, all differences between all treatment levels were not significant with regard to the PI curve parameters $rETR_{max}$ and I_k [$rETR_{max}$ and I_k, $F(1,39)$, $p > 0.05$; Table 3]. High temperatures decreased NPQ_{max} significantly to 1.86 as compared to 2.81 at ambient temperatures [NPQ_{max}, $F(1,39)$, $p = 0.014$; Table 3; Fig. 3b].

Genotypic diversity level effect

In autumn, the highest diversity levels tended to feature the highest survival of all treatments, but this trend was not significant [diversity level, $F(2,11) = 0.70$, $p = 0.517$; Fig. 4a; Table 4]. In winter, lowest survival (20.07 ± 14.63 %) was observed in the lowest diversity level under high-temperature and acidified conditions, whereas highest survival was measured in the intermediate diversity level under high-temperature conditions (ca. 76.03 ± 6.03 %; Fig. 4b). Under increased temperatures, high diversity levels also showed higher survival than the low diversity level [diversity level, $F(2,11) = 4.1$, $p = 0.0467$; Fig. 4b; Table 4]. There was no diversity level effect with regard to growth in any time period under any treatment (diversity level, p value >0.05; Table 5).

Table 3 Results of mixed-model ANOVA with the two fixed factors temperature and CO_2 and the random factor sibling group on chlorophyll *a* fluorescence parameters measured at the end of the experiment (March 2014) on *F. vesiculosus* germlings

Source of variation	numDF	denDF	*F* value	*p* value
(a) Maximum relative electron transport rate ($rETR_{max}$)				
Temp	1	39	1.033	0.316
CO_2	1	39	0.364	0.549
Temp × CO_2	1	39	0.254	0.617
(b) Light saturation coefficient (I_k)				
Temp	1	39	0.020	0.888
CO_2	1	39	0.338	0.564
Temp × CO_2	1	39	0.061	0.807
(c) Non-photochemical quenching (NPQ_{max})				
Temp	1	39	6.522	0.015
CO_2	1	39	2.058	0.159
Temp × CO_2	1	39	0.452	0.506
(d) Maximum quantum yield (F_v/F_m)				
Temp	1	35	1.159	0.289
CO_2	1	35	3.977	0.054
Temp × CO_2	1	35	1.658	0.206

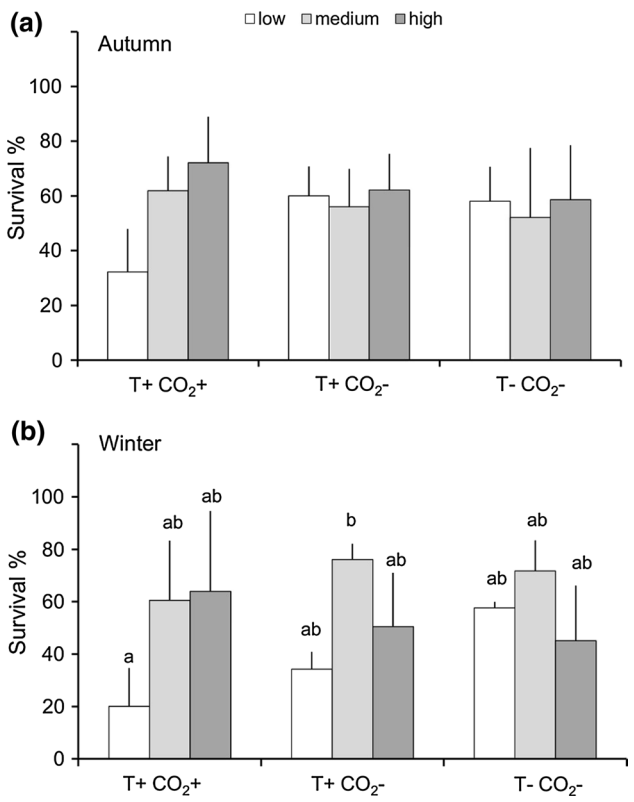

Fig. 4 Survival (%) of the different diversity levels: low (*white bars*), medium (*grey bars*) and high (*dark-grey bars*) at three treatments in **a** autumn and **b** winter. Mean values +SD are shown ($n = 3$). Different letters above the bars indicate significant differences (p value <0.05) between the treatments after Tukey's test

The variance in survival ($\%^2$) between groups of the low diversity level was high compared to the variance between groups of medium and high diversity levels during autumn and winter (Fig. 5a, b). The high variance of single sibling groups was present at all treatments except in winter under high-temperature and non-acidified conditions, where groups of the median diversity level showed highest variance (Fig. 5b). The response variability among experimental populations tended to decrease with increasing genetic diversity level. In 5 out of 6 treatment/season combinations, genetic diversity explained more than 60 % of response variability.

Sibling group differences

Sibling groups (1–8) showed strong differences in their sensitivities with regard to survival (%) under high-temperature and high-pCO_2 conditions. Differences between some of the sibling groups were significant in summer and winter [sibling group, in summer $F(7,16) = 2.479$, $p = 0.026$ and in winter $F(7,16) = 2.478$, $p = 0.029$; Table 5a). The single and combined effects of high-temperature and high-CO_2 treatments on survival were beneficial for some of the groups but prejudicial for other groups: high-temperature treatment enhanced survival in one sibling group but decreased survival in six sibling groups compared to ambient temperatures. Acidification led to increased survival of six sibling groups but to decreased survival in two sibling groups compared to ambient CO_2 conditions. The combined effect of high temperature and high CO_2 increased survival of three sibling groups but caused lower survival of five sibling groups compared to ambient conditions. There were no significant differences in spring and autumn [sibling group, in spring $F(7,16) = 1.169$ $p = 0.324$ and in autumn $F(7,16) = 2.478$, $p = 0.338$]. The interaction of both factors, temperature and CO_2, seemed to be mainly antagonistic (regarding germlings' mortality) on all sibling groups in spring and in summer. Sibling group variation was also high with regard to growth rates (% d^{-1}) during all seasons. However, differences in growth rates were significant only in spring [sibling groups, $F(7,16) = 1.373$, $p = 0.014$; Table 5].

Discussion

The sensitivity of *Fucus vesiculosus* germlings to warming was higher than towards acidification, although responses to both factors are highly season dependent. Accordingly, warming had positive effects on growth in early summer but caused severe mortalities in late summer. Also the photophysiological parameter NPQ_{max} decreased under warming, while acidification did not have an effect on this

Table 4 Results of the mixed-model ANOVA testing the two fixed factors diversity level (Div.) (three levels: low, medium and high) and treatment (three levels: T+CO_2+, T+CO_2− and T−CO_2−) including the random factor sibling group on *Fucus vesiculosus* germlings regarding (a) survival (arcsin-transformed) and (b) relative growth rates (% d^{-1})

Source of variation	numDF	denDF	Autumn		Winter	
			F value	*p* value	*F* value	*p* value
Survival						
Treatment	2	106	0.737	0.481	3.125	0.048
Div	2	11	0.701	0.517	4.101	0.047
Treatment × Div	4	106	2.108	0.085	2.524	0.045
Growth rate						
Treatment	2	73	28.509	<0.001	2.135	0.126
Div	2	7	0.539	0.606	0.299	0.750
Treatment × div	4	73	0.498	0.738	1.928	0.115

Table 5 Results of three-way ANOVA for each season showing differences between sibling groups testing the factors: temperature, CO_2 and sibling group (sib) in (a) survival (arcsin-transformed) and (b) relative growth rate (% d^{-1})

Source of variation	Df	*F* value	*p* value	*F* value	*p* value
(a) Survival					
		Spring		Summer	
Temp	1	0.025	0.876	12.507	<0.001
CO_2	1	1.167	0.284	1.879	0.175
Sib	7	1.184	0.324	2.479	0.026
Temp × CO_2	1	0.010	0.921	0.233	0.631
Temp × sib	7	0.441	0.873	1.816	0.099
CO_2 × sib	7	0.320	0.942	0.754	0.628
Temp × CO_2 × sib	7	0.951	0.474	1.660	0.135
Residuals	64				
		Autumn		Winter	
Treatment	2	3.600	0.035	6.311	0.004
Sib	7	1.169	0.338	2.478	0.029
Treatment × sib	14	0.849	0.615	1.496	0.150
Residuals	48				
(b) Growth rate					
		Spring		Summer	
Temp	1	3.886	0.054	5.875	0.019
CO_2	1	12.193	<0.001	0.246	0.622
Sib	6	2.960	0.014	0.466	0.830
Temp × CO_2	1	3.306	0.075	0.950	0.334
Temp × sib	6	1.247	0.297	1.084	0.385
CO_2 × sib	6	2.377	0.041	0.580	0.745
Temp × CO_2 × sib	6	1.550	0.179	0.992	0.441
Residuals	50				
		Autumn		Winter	
Treatment	2	10.953	<0.001	7.631	0.001
Sib	7	1.373	0.239	1.640	0.147
Treatment × sib	14	1.916	0.049	1.863	0.056
Residuals	46				

parameter. The variability in the responses of the sibling groups to warming and acidification was high, supporting the hypothesis that the tolerance against environmental stress is increased in populations of higher genetic diversity. Corroborating this, survival of germlings increased under warming in the high diversity level compared to the

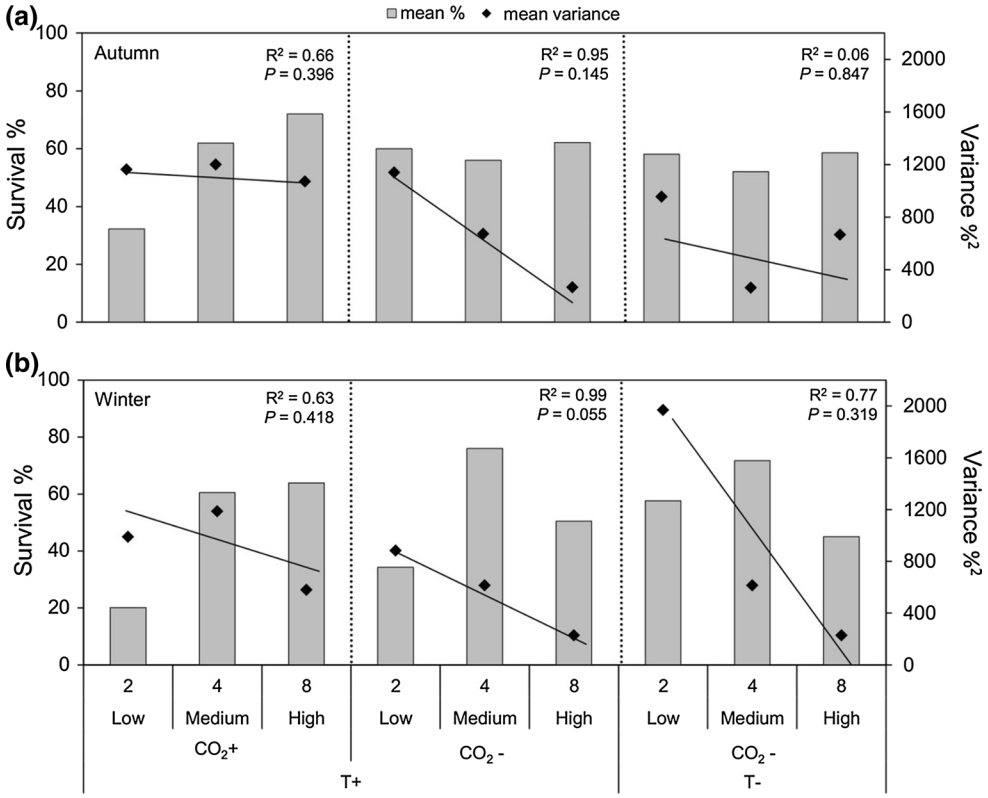

Fig. 5 Variances ($\%^2$) between the groups of each of the three diversity levels (low: single sibling groups, medium: pairs, high: quartets) with 2, 4 and 8 parents each diversity level, respectively, for survival (%). *Bars* show means ($n = 3$) for each combination of diversity and treatment level in **a** autumn and **b** winter. Trend lines for the variances within each treatment level are shown as well as the determination coefficient R^2

low diversity level, supporting the hypothesis that the tolerance against environmental stress is increased in populations of higher genetic diversity. Macroalgal germlings, growing under the environmental gradients of the Baltic shallow subtidal, are regularly subjected to strong fluctuations of temperature and pCO_2 (Saderne et al. 2013). This, over time, may have led to a preadaptation to global changes (e.g. Pansch et al. 2014). However, in the near-natural environment of the KOB, so far unknown sensitivities were revealed regarding the interactive effects of temperature, acidification and season. Responses in physiological performance of *F. vesiculosus* germlings to warming were positive until July as reflected in enhanced growth rates and are consistent to those of adult *F. vesiculosus* in the northern Baltic proper (Lehvo et al. 2001). Enhanced growth rates under warm conditions may be explained by stimulated metabolic processes according the Q10-rule (Nygård and Dring 2008; Nielsen et al. 2014). During a natural heat wave in August, however, temperatures reached >27 °C (Online Resource 2) exceeding the thermal tolerance range of juveniles of this species (Li and Brawley 2004; Maczassek 2014) as reflected in a severe mortality. These results indicate that future ocean warming effects on survival are strongest during summer, differing among seasons. As a consequence of warming, local extinctions and range shifts of *F. vesiculosus* were predicted for the next century by Jueterbock et al. (2013) and were observed for other seaweed species (Wernberg et al. 2011).

Warming-induced mortality was also observed in winter, when twice as many germlings died under warming and acidification compared to ambient conditions. This seems surprising, considering that the increase in temperatures ranged from ambient 3–7 °C to only experimental 8–12 °C (December to January), which, at least for adults, is towards the optimum temperature range of *F. vesiculosus* (Graiff et al. 2015). Warming during winter may enhance all metabolic processes (e.g. respiration), while photosynthesis is still limited due to low-light conditions (Rohde et al. 2008). In winter the daily dose of irradiation is probably below the light compensation point of the *F. vesiculosus* germlings (Wahl et al. 2011) (Online Resource 3). Consequently, the resulting light limitation may have biased towards heterotrophic processes. Therefore, it is assumed that the accelerated metabolism under warming has provoked an overexploitation of storage products and hence a lethal energy debt. The interactive effect of temperature and light might be relevant for early life-stages of seaweeds which often occur in the shaded understory vegetation.

Acidification affected growth and survival of the germlings less strongly than warming, but high pCO_2 increased survival in spring and growth in late summer. The reason for this is either an enhanced carboxylation at high-pCO_2 conditions as previously reported for other algae

(Olischläger et al. 2012, 2013; Koch et al. 2013; Saderne et al. 2013) or down-regulation of energy-consuming carbon-concentrating mechanisms (CCMs) under these conditions (Beardall and Giordano 2002; Wu et al. 2008).

Under ambient conditions, germlings seem to require less light than adults possibly reflecting an adaptation to their natural shaded habitat, i.e. the understory of adults. The photosynthetic performance, expressed as relative electron transport rate and light saturation point, of *F. vesiculosus* germlings under ambient conditions was only half compared to adults (A. Graiff, unpublished results). The photosynthetic apparatus of juveniles may not be fully developed and hence be more sensitive to environmental stress. The maximum quantum yield (F_v/F_m) of the germlings under warming at the end of the winter experiment (March 2014) was not reduced, but they showed a significantly decreased non-photochemical quenching (NPQ_{max}) compared to ambient temperatures (Fig. 3b). Lower NPQ_{max} and reduced $rETR_{max}$ under the high-temperature treatment can be explained by the presence of temperature sensitive enzymes of photophosphorylation and the stability of PSII (e.g. D1 protein), as reviewed by Allakhverdiev et al. (2008). Non-photochemical quenching is a proxy of xanthophyll pigment cycling, which protects photosystems from overexcitation (Lavaud et al. 2002a, b; Ruban et al. 2007). Therefore, data on non-photochemical quenching regulation better reflect the actual physiological state of algae under stress than other chlorophyll *a* fluorescence parameters, indicating the decreased capacity of *F. vesiculosus* germlings for stress resistance under warmed conditions.

Chlorophyll *a* fluorescence parameters from *F. vesiculosus* germlings were not significantly influenced by acidification neither under ambient nor under elevated temperatures. In contrast, stimulating effects of increasing external DIC on the photosynthesis and relative electron transport rates of adult brown algae have been reported, possibly due to increased activity of RuBisCO (Forster and Dring 1992; Nygård and Dring 2008; Johnson et al. 2012). In particular, the kelps *Laminaria digitata* and *Saccharina latissima* as well as Baltic *F. vesiculosus* responded to moderately increased DIC with elevated rates of photosynthesis and carbon acquisition (Schmid et al. 1996; Klenell et al. 2004; Nygård and Dring 2008). It is assumed that photorespiration is reduced under elevated pCO_2 and less energy is required for recharging the internal carbon storage after periods of high photosynthetic activity. However, these beneficial effects of increased pCO_2 on photosynthetic performance on *F. vesiculosus* germlings may have been masked by the interaction with other fluctuating abiotic factors (e.g. nutrients, irradiances) as was previously reported for other non-calcifying algae (Sarker et al. 2013).

F. vesiculosus germlings in populations with higher genetic diversity tended to survive better under warmer conditions than those in the low diversity level. These findings support that populations with higher genetic diversity are more resilient towards environmental stress than those of lower genetic diversity. Ongoing genetic analysis will reveal the causes for this observed pattern; hence, for the moment all interpretation is speculative. Possibly, neighbour effects exist that are more beneficial for germlings if their neighbours are more different among each other than full-sibs would be. There are several possible ways how diverse neighbouring germlings could have influenced each other positively, in analogy to processes and complementarity found in species-rich communities and modelled in ecosystem functioning concepts (Hooper et al. 2005). *F. vesiculosus* genotypes vary in their capacity of antifouling defence strength and in the release of defence metabolites (Honkanen and Jormalainen 2005). Higher genetic diversity level may present well-defended genotypes that protect more weakly defended neighbours [associational defence sensu Wahl and Hay (1995)]. Hence, antifouling capacity is indeed crucial for seaweed fitness in general (da Gama et al. 2014) and may be particularly important for early ontogenetic stages (Wahl et al. 2011). Facilitation and niche differentiation under warming have been observed in other species, as among different genotypes of the eelgrass *Zostera marina* (Reusch et al. 2005), where complementarity dominated over selection processes, as well as improved settlement success in the early life-stage of the barnacle *Balanus improvisus* (Gamfeldt et al. 2005). The higher resistance conferred by intraspecific variability for *F. vesiculosus* to environmental stress underpins the increasing plea for the inclusion of intraspecific variability in ecosystem functioning concepts (Reusch and Hughes 2006). In fact, there is a growing recognition of including the within-species variation when using an upscaling approach: the genotypic variability determines the width of species niches and has therefore implications on an interspecific level and on the community structure (Violle et al. 2012).

In conclusion, our study highlights the importance of evaluating the interactive impacts of multiple stressors (Folt et al. 1999) in the context of variable environmental conditions (seasons), natural fluctuation (Wahl et al. 2015c) and variable intraspecific diversity (Reusch and Hughes 2006; Reusch and Wood 2007; Pauls et al. 2013). Our results also show the strong pressure on early life-stages of *F. vesiculosus* under climate change conditions, mainly caused by warming during summer. Sufficient genetic diversity in the population, however, provides a strong stress-driven selection which might quickly enhance tolerance to global change. Although the observed antagonistic interaction between temperature and pCO_2 may mitigate high impacts of global change variables, further genetic analyses are

required for understanding the exact significance. The past massive retreat of this brown alga in the Baltic Sea indicates that additional abiotic or biotic stressors such as increased grazing, overfishing or fouling in combination with the inherently low genetic variation of *F. vesiculosus* are responsible for this *quasi* collapse. Filling these knowledge gaps will help to better predict the fate of *F. vesiculosus* in the Baltic Sea.

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