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RESEARCH ARTICLE

Sequential disturbances alter the outcome of inter-genotypic interactions in a clonal plant

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Abstract

1. Multiple disturbances can have mixed effects on biodiversity. Whether the interaction of sequential disturbances drives local extinctions or promotes diversity depends on the severity of biomass reductions relative to any stabilizing and/or equalizing effects generated by the disturbance regimes.
2. Through a manipulative mesocosm experiment, we examined how warming events in the fall and simulated grazing disturbance (i.e. clipping) in the winter affected the density, biomass and genotypic diversity of assemblages of the clonal seagrass *Zostera marina*.
3. We show that the interaction of the two disturbance types reduced density and biomass to a greater degree than warming or clipping alone.
4. The genotype with the highest biomass in the assemblage shifted under the different experimental regimes such that the traits of winners were distinct in the different treatments. The favouring of different traits by different disturbances led to reduced evenness when a single disturbance was applied, and enhanced evenness under multiple disturbances.
5. We conclude that sequential disturbances can alter the outcome of inter-genotypic interactions and maintain genotypic diversity in clonal populations. Our study expands the context in which disturbance can influence intraspecific diversity by showing that fluctuating selection may result from the sequential application of different disturbance types and not simply seasonal changes in a single agent.

KEYWORDS
disturbance, diversity, eelgrass, fluctuating selection, genotypic interactions, grazing, warming

1 | INTRODUCTION

Multiple disturbances can have contrasting or interactive effects on biodiversity. When disturbances result in reductions in abundance beyond the ability for a species to recover, regime shifts or local extinctions may result (reviewed in Buma, 2015; Paine et al., 1998; Turner, 2010). Disturbance can also affect diversity by reducing the average fitness differences between species, which reduces the impact of competition and delays (but does not prevent) exclusion (Chesson, 2000). However, the occurrence of multiple different types of disturbances can help promote the long-term coexistence of species if they create opportunities for niche differentiation in space or time (e.g. Chesson, 2000; Chesson & Huntly, 1997). This occurs if the different disturbances favour alternative ecological strategies such that the existence of multiple disturbances has a stabilizing effect on diversity (sensu Chesson, 2000), for example by creating a fluctuating environment in which no species is favoured for long enough to achieve

dominance (e.g. Hutchinson, 1961; Miller & Chesson, 2009). Predicting whether disturbance will maintain diversity or result in exclusion depends on the balancing roles disturbance plays in altering competition among species through equalizing and/or stabilizing mechanisms while also decreasing survival rates within a species (Chase et al., 2002; Chesson & Huntly, 1997).

Similarly, the fluctuating environmental conditions caused by varying disturbance regimes can influence the maintenance of genetic diversity (Banks et al., 2013; Davies et al., 2016; Fraser et al., 2018; Hughes et al., 2007). For example, reducing herbivory on evening primrose increased evenness and shifted the identity of dominant genotypes away from those that are resistant to herbivory and towards earlier flowering genotypes that are more tolerant of competition (Agrawal et al., 2012). Disturbance–diversity relationships are particularly relevant for species that grow clonally as asexual propagation maintains a genotype's distinct phenotype and allows for interactions among individuals within a population to play out in analogous ways to species acting within a community (reviewed by Hughes et al., 2008; Vellend & Geber, 2005). Observational surveys of clonal plants provide mixed evidence regarding the correlation between genotypic diversity and disturbance (e.g. McMahon et al., 2017; Reisch & Scheitler, 2009; Reynolds et al., 2019; Rusterholz et al., 2009; Yu et al., 2019), and disentangling the underlying mechanisms driving variance in this relationship remains an important avenue of research (Banks et al., 2013). Manipulative field experiments can assess the effects of varying disturbance regimes on the recruitment of new individuals (Herrera & Bazaga, 2016; Hidding et al., 2014; Macreadie et al., 2014; Peng et al., 2015; Reusch, 2006; Veeneklaas et al., 2011) but sampling at a fine enough scale to capture the effects of disturbance on altering the outcome of inter-genotypic interactions is challenging. Conversely, laboratory micro- and mesocosms with known genotypes can isolate the effects of disturbance on inter-genotypic interactions while removing the potentially confounding effects of variation in recruitment (e.g. Weider, 1992).

Populations of the seagrass *Zostera marina* (hereafter *Zostera*) have served as a model system for research on the ecological consequences of genetic diversity (e.g. Hughes & Stachowicz, 2004, 2009; Reusch et al., 2005; Williams, 2001) and by extension provide an opportunity for studying the effect of disturbances on genotypic coexistence. *Zostera* occurs in temperate coastal ecosystems of the Northern hemisphere. Like all seagrasses, *Zostera* reproduces both sexually via seed production and through clonal propagation. In northern California, populations of *Zostera* are genetically diverse (Hughes & Stachowicz, 2009; Kamel et al., 2012; Olsen et al., 2004), this genetic diversity is stable across time (Reynolds et al., 2017), and meadows can have upwards of four unique genotypes interacting within a 10 cm × 10 cm area (Abbott & Stachowicz, 2016). *Zostera* genotypes differ phenotypically in common gardens (Abbott et al., 2018; Hughes et al., 2009) and competition can lead to the exclusion of genotypes at the local scale (Abbott & Stachowicz, 2016). However, the effects of disturbance in mitigating competitive exclusion among eelgrass genotypes remain unclear (Reusch, 2006).

Zostera meadows in northern California experience multiple stresses and disturbances within any given year, which may contribute to the maintenance of the observed genetic diversity. From 2014 to 2016, a marine heatwave (i.e. 'warm blob') in the NE Pacific (Gentemann et al., 2017) exposed *Zostera* populations in Bodega Harbor, CA to temperature anomalies between 2°C and 4°C above the climatic mean (Sanford et al., 2019). Experimental mesocosms simulating this warming event revealed that the negative effects of warming events on *Zostera* productivity are often delayed and prolonged (Reynolds et al., 2016), individual genotypes vary in their sensitivity to warming (DuBois et al., 2019; Reynolds et al., 2016), the relative performance of genotypes shifts after warming events (DuBois et al., 2019), and warming can alter *Zostera* morphology with transgenerational consequences to clonal offspring (DuBois et al., 2020). Seasonal, but less extreme, warming occurs each year in late summer and early fall (e.g. Reynolds et al., 2016).

The stress from warming could exacerbate the susceptibility of *Zostera* populations to additional disturbances. Along the western coast of North America, the warmest temperatures of the year are followed by winter season grazing by migratory Pacific Black Brant geese, *Branta bernicla nigricans*. Grazing disturbance has clear and direct effects on seagrass above-ground biomass as the geese remove the leaf tissue (Ganter, 2000, reviewed in Kollars et al., 2017). However, because grazing does not damage the basal meristem, it rarely causes mortality of individual shoots and plants may regrow leaf tissue after a grazing event (e.g. Ganter, 2000; Moore & Black, 2006; see Hulme, 1996 for a general discussion of defoliation as a disturbance event). Despite the ability to regenerate tissue, clipping experiments with *Zostera* have revealed reductions in shoot density in clipped treatments relative to controls, likely due to a reduced ability to generate new shoots while resources are diverted to replacing lost tissue (e.g. Hughes, 2006; N.M. Kollars & J.J. Stachowicz, unpubl. data; Ruesink et al., 2012).

Here we tested how warming interacts with simulated grazing to affect the density, biomass and maintenance of genotypic diversity in *Zostera*. We used outdoor mesocosms with natural flow-through seawater and experimentally mimicked the natural seasonal timing of the disturbances by exposing pairs of *Zostera* genotypes to a factorial combination of elevated temperature in fall followed by simulated goose grazing (i.e. clipping) in winter. We predicted that warming and clipping would each independently reduce *Zostera* density and biomass and that the interaction of the two disturbance types would intensify these reductions beyond additive expectations. We also predicted that warming and clipping would alter the identity of the winning genotype within the pair due to selection for genotypes with different phenotypes (e.g. Agrawal et al., 2012). As a result, we expected that the combination of warming and clipping would increase evenness within the pair by creating fluctuating selection compared to more consistent selective effects in warming or clipping alone.

2 | MATERIALS AND METHODS

Zostera genotypes used in our experiments were collected from Bodega Harbor, CA and cultured in outdoor, flow-through seawater tanks at the Bodega Marine Laboratory (Abbott et al., 2018; Hughes et al., 2009). It total, we used 39 genotypes to randomly generate 50 unique pairings (see Table S1 for a list of the genotypes used in each pair). We grew one replicate of each pair under each of the treatments. We chose to maximize the number of unique parings over replicating specific pairings to better model how multiple disturbances could affect interaction outcome among genotypes independent of the identity of specific genotypes. We harvested genotypes from cultured stocks in mid-August of 2017. We measured the initial shoot length (first rhizome node to longest leaf), rhizome diameter, and rhizome length for each shoot, but standardized the rhizome length to be no longer than 6 cm. For each pair, we planted a single shoot of each genotype in a square plastic flowerpot (8.89 cm^3) filled with sieved and homogenized sediment collected from Bodega Harbor. We loosely tied a piece of coloured flagging tape around the base of the terminal shoot of each genotype to mark its identity.

For the first part of the experiment, we grew the plants in 20 outdoor flow-through seawater tanks (60 cm L × 30 cm W × 60 cm H; a volume of 113 L; flow rate approximately 60 L/hr) at the Bodega Marine Laboratory (see also DuBois et al., 2020; Reynolds et al., 2016). In all, 10 tanks received seawater at ambient temperature and 10 received seawater passed through a sump tank with titanium heaters (Process Technologies 1000W immersion heaters). Ambient tanks held replicates for the control and clipping treatments and heated tanks held replicates for the warming and warming + clipping treatments. We placed five pairs in each tank across the two experimental temperature regimes, which resulted in a total of 10 pots per tank. We recorded temperature in each tank at 15-min intervals using Onset Hobo Pendant Temperature/Light 64K Data Loggers.

We allowed the plants to recover from transplantation for 7 weeks prior to applying the warming treatment. Plants in both the control and warmed treatments were at the same temperature during this recovery period. We then increased the temperature in the warmed treatments by 3.4°C relative to the ambient control for 40 days (see Figure S1 for the full temperature profile during this time). This temperature increase is similar in magnitude and duration to temperature increases during recent summer/fall heat waves (e.g. Reynolds et al., 2016; Sanford et al., 2019). Immediately after turning off the heat, we counted the number of shoots in each pot. Three weeks after turning off the heat, we transferred the pairs from the experimental warming mesocosms into larger pots (11.4 cm diameter × 9.5 cm H) filled with freshly collected and sieved sediment from Bodega Harbor to allow for the increase in clonal size we expected as ramets expanded over the remainder of the experiment. These larger pots were placed into a single tank (3.68 m D × 0.7 m H; approximately 7,450 L) with flow-through seawater supplied at a rate of approximately 1,500 L/hr through irrigation tubing placed around the tank perimeter.

We allowed the plants to recover from transplantation to the larger pots for 7 weeks before implementing the clipping treatment in late January of 2018. To mimic grazing by Brant geese, we used scissors to remove the leaf tissue for all the shoots in the pots assigned to the clipping treatments. Brant will subsequently graze the young and nutritious re-growth from previously grazed shoots (Moore & Black, 2006) and to mimic this we clipped plants a second time, 6 weeks after the first clipping. We counted the total number of shoots in the pot before each round of clipping.

We concluded the experiment in late April of 2018, 6 weeks after the second clipping, which allowed surviving shoots time to re-grow clipped biomass. We removed the plants from the pots and carefully separated the genotypes using the remaining tags and intact rhizome connections. For ramets that we could not unambiguously trace to a tag, we genotyped the sample at 11 microsatellite loci, and compared the ramet's multi-locus genotype to the known genotypes in the pair (see Abbott et al., 2018 and references therein for details on methods). Using this method, we were able to assign most ramets present at the end of the experiment to one of the two planted genets ($n = 5$ pots with unassigned ramets due to failed PCR and thus removed from analysis). After removal from the pot, we counted the number of shoots for each genotype and measured the mass of above-ground and below-ground tissue after drying to constant mass at 60°C.

2.1 | Statistical analyses

2.1.1 | Aggregate (pot level) density and biomass

First, we tested the effect of single and sequential disturbances on aggregate plant density and biomass using pot-level data (summed abundance of both genotypes). Specifically, we used GLMs to test the effect of warming, clipping and their interaction on the total number of shoots in a pot at each stage of the experiment (immediately post-warming, 10 weeks post-warming, 6 weeks after the first clipping and at breakdown) and the response of the final pot-level biomass. We separated the analysis by stage because our interest was in comparing treatment outcomes within a time point and not across time, especially considering that treatment application confounded time in our design (e.g. clipping did not occur until the second half of the experiment). We initially modelled each of the shoot count data variables with a Poisson error distribution but used a quasi-Poisson distribution if the data showed overdispersion (tested in the AER package; Kleiber & Zeileis, 2008; see also Table 1). For the biomass variables, we chose a gamma distribution to restrict the model to positive integers. In each case, we followed fitting the GLM with analysis of deviance using the ANOVA function to generate *F*-ratios and *p* values. We performed these and the proceeding analyses in R version 3.5.3 (R Core Team, 2020). We removed from analysis any pot in which either one genotype died prior to treatment application, no shoots survived, or human error (i.e. misapplication of treatment, unidentified ramets and

TABLE 1 Analysis of deviance results from GLMs testing the effects of warming events and clipping disturbance on *Zostera marina* density and biomass variables. $p \leq 0.05$ are in bold

Response	Distribution	Factor	Dev	Res *df*	Res Dev	*F* (*p* value)
Shoot counts immediately after warming	Poisson: Log link	NULL		134	60.572	
		Warming	0.01	133	60.561	(0.92)[a]
		—	—	—	—	—
		—	—	—	—	—
Shoot counts 10 weeks after warming	Quasi-Poisson: Log link	NULL		142	228.33	
		Warming	9.75	141	218.58	**6.96 (<0.01)**
		—	—	—	—	—
		—	—	—	—	—
Shoot counts after first clipping	Quasi-Poisson: Log link	NULL		142	428.28	
		Warming	26.83	141	401.45	**10.13 (<0.01)**
		Clipping	15.87	140	385.58	**5.99 (0.02)**
		Interaction	1.60	139	383.97	0.61 (0.44)
Shoot counts at breakdown	Quasi-Poisson: Log link	NULL		142	1,232.53	
		Warming	31.53	141	1,211.00	**4.59 (0.03)**
		Clipping	299.11	140	911.89	**43.51 (<0.01)**
		Interaction	14.48	139	897.41	2.11 (0.15)
Above-ground Biomass	Gamma: Inverse link	NULL		136	238.86	
		Warming	1.67	135	237.19	1.77 (0.19)
		Clipping	93.01	134	144.10	**98.71 (<0.01)**
		Interaction	2.13	133	141.96	2.26 (0.13)
Below-ground Biomass	Gamma: Inverse link	NULL		137	147.99	
		Warming	1.69	136	146.30	2.96 (0.09)
		Clipping	43.78	135	102.53	**76.59 (<0.01)**
		Interaction	4.22	134	98.31	**7.38 (<0.01)**

Abbreviations: Dev, deviance; *df*, degrees of freedom; Res, residual.

[a]The analysis of deviance for the GLM modelling shoot counts immediately after heating used a chi-squared test instead of a *F*-test.

labelling errors). The experiment concluded with at least 30 replicates per treatment (Table S1).

2.1.2 | Interaction outcome between genotypes

To assess treatment effects on the interaction outcome between genotypes, we considered the relative abundance of each genotype within the pair and assessed how treatments affected (a) the identity and traits of the more abundant genet and (b) the evenness of the abundances of the two genets (see below for a description of how we measured evenness). We used below-ground biomass as our metric for abundance because the clipping application purposefully reduced the above-ground biomass during the experiment and thus might not provide a reliable indicator of the relative success of the individuals in a pot. Previous studies have also shown that below-ground storage is a strong predictor of a plant's ability to recover from above-ground stress (e.g. Alcoverro et al., 1999; Govers et al., 2015, reviewed in Thomas et al., 2017) and can be influenced by the genotypic identity of a neighbouring plant (e.g. Genung et al., 2012). Therefore, we considered below-ground biomass to be a product of both the genet's physiological response to the disturbance treatment and competition within the pair.

We first tested whether the identity of the genotype with the highest below-ground biomass in each pair varied across the treatments. Given the relationship between biomass production and competitive outcomes (e.g. Gaudet & Keddy, 1988), we labelled the genotype with the highest relative abundance of below-ground biomass as the 'dominant' genotype within that pair. Next, we calculated the relative abundance of this same genotype across each of the four treatments: shifts in the relative abundance among treatments indicated that the relative dominance changed in that treatment with respect to the control. Because relative abundance is a proportional variable and bounded between 0 and 1, we did not analyse this response using linear models that require a specified error distribution. Instead, we used non-parametric permutation tests with 1,000 permutations within an ANOVA framework and with treatment as a factor (sensu Anderson, 2001). We permuted the *F*-statistic and calculated a *p* value using one-tailed tests of the null hypothesis that the observed and simulated *F*-statistic come from the same distribution (as described by Gotelli & Ellison, 2004). We followed this analysis with post-hoc

pairwise comparisons using the pairwise PermutationTest function in the RCOMPANION package (S. Mangiafico); we adjusted *alpha* for multiple comparisons according to Benjamini and Hochberg (1995).

We found that the identity of the more abundant genotype within the pair changed across treatments (see Section 3). We tested whether this shift was predictable based on traits known to influence relative performance among genotypes of *Zostera* as a function of temperature (DuBois et al., 2019; Reynolds et al., 2016) and clipping (N.M. Kollars & J.J. Stachowicz, unpubl. data). We compared the mean trait state of the genotypes in each of the treatments using previously measured trait values (Abbott et al., 2018). Though both source studies for our genotypes measured traits in a common garden setting (Abbott et al., 2018; Hughes et al., 2009), the experiments were conducted under different ambient conditions and thus not comparable. Therefore, for trait-based analyses, we chose to only retain replicates in which both genotypes in the pair were also studied in Abbott et al. (2018), which characterized traits for the majority of our genotypes (final replicate size: 15–23 pairs; see Table S1). Focal traits included the following: (a) photosynthetic efficiency (measured as alpha, the initial slope of the rapid light curve and a metric characterizing the efficiency of photon capture when light is limiting), (b) maximum electron transport rate (ETR_{max}; measured as the maximum number of electrons moving through photosystem II when light is saturating) and (c) above-ground versus below-ground resource allocation (measured as the above-ground to below-ground biomass ratio). See Abbott et al. (2018) for details on how each trait was measured. Functionally, higher values of photosynthetic efficiency and the maximum electron transport rate (ETR_{max}) increase the potential for carbon fixation, while the above-ground to below-ground biomass ratio represents the relative investment of plants to tissue available for photosynthesis (above-ground biomass) versus storage and nutrient acquisition (below-ground biomass). We calculated a multivariate trait index for the three focal traits and for each genotype using principal component analysis (PCA; generated with the prcomp function in base R) using standardized trait values. We visualized the differences among treatments by plotting the mean multivariate trait value of PC1 against PC2 for the genotype within a pair whose relative abundance (based on below-ground biomass) fell into three categories: >0.7, between 0.3 and 0.7 or below 0.3. We subjectively classified these interaction outcome categories as 'winning', 'coexisting' or 'losing', respectively.

Finally, we examined the overall effects of treatment on evenness across all experimental pairs. Because there were only two genotypes in a pot, their relative abundances must sum to 1 and maximum evenness occurs when both genotypes have a relative abundance of 0.5. As above, we calculated relative abundance based on below-ground biomass. We used relative abundance of the less abundant genotype within a pair as our proxy for genotypic evenness such that a value of 0 would indicate exclusion from the pot and a value of 0.5 would indicate that genotypes had equal biomass. We tested for the effects of warming, clipping and their interaction using permutation analyses within an ANOVA framework (see above).

3 | RESULTS

3.1 | Aggregate (pot level) density and biomass

Both the warming and clipping treatments reduced the total number of shoots in the pot throughout the experiment and the effects of these appeared to be mostly additive (Figure 1a). Warming reduced

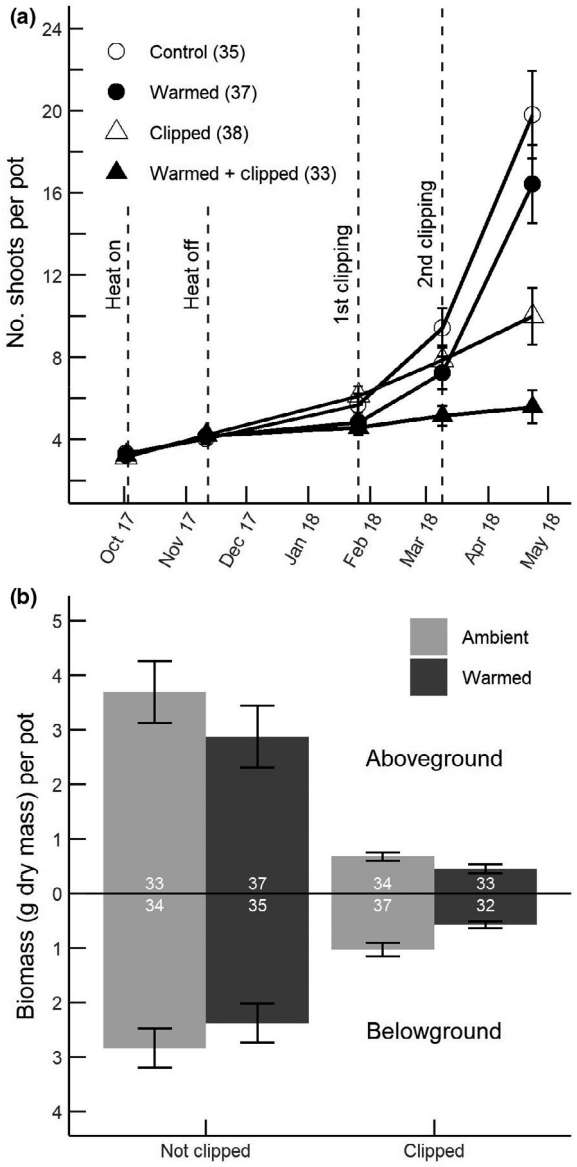

FIGURE 1 The response of *Zostera marina* biomass to a warming event followed by a clipping disturbance. (a) Total number of shoots in the pot through time. Dashed lines indicate the timing of treatment application. Open circles: control; closed circles: warmed; open triangles: clipped; closed triangles: warmed + clipped. Numbers in parentheses are sample size. (b) The response of total above-ground biomass and below-ground biomass at the experimental breakdown. Light grey colour: ambient temperature treatment; dark grey colour: warmed temperature treatment. Numbers in white are sample size. For both (a and b), *y*-error bars represent standard error of the mean. See Table 1 for statistical analysis

shoot counts within 10 weeks after the return to ambient temperature ($F = 6.96$, $p < 0.01$; Table 1) and this effect persisted throughout the experiment, reaching a maximum of 17% reduction in shoot density by the end of the experiment (22 weeks after the warming event; $F = 4.59$, $p = 0.03$; Table 1). Clipping reduced shoot density within 6 weeks after the first application (9.1% reduction in the clipping treatment and 29.3% reduction in the warming + clipping treatment relative to the control; $F = 5.99$, $p = 0.02$; Table 1). Two clipping applications reduced the mean total number of shoots in the pot by 49.5% in the clipping treatment and 71.8% in the warming + clipping treatment (clipping effect: $F = 43.51$, $p < 0.01$; Table 1), but there was no interaction between warming and clipping ($F = 2.11$, $p = 0.15$; Table 1).

In contrast, the effect of clipping on total below-ground biomass in a pot depended on warming (warming × clipping interaction: $F = 7.38$, $p < 0.01$; Table 1). The warming + clipping treatment showed a 79.6% reduction in mean below-ground biomass relative to the controls, while the clipping only treatment reduced mean below-ground biomass by 63.6% and warming alone had no influence (Figure 1b). Not surprisingly, clipping (which involved direct removal of above-ground biomass) exerted a dominant influence on above-ground biomass (clipping effect: $F = 98.7$, $p < 0.01$; Figure 1b; Table 1), with no effect of warming or the interaction between warming and clipping (Table 1).

3.2 | Interaction outcome between genotypes

The identity of the dominant genotype (relative abundance > 0.5) varied among treatments. To visualize this, we plotted the relative abundance of the dominant genotype in unmanipulated pots relative to when it was grown in the same pair combination in each of the other treatments (Figure 2, $F = 4.34$, $p < 0.01$; see also Figure S3). The warming + clipping treatment reduced the mean relative abundance of the genotype that had dominated in the control from 77.8% to 49.9% (adjusted $p < 0.01$). Although the dominant genotype in both the warming only and clipping only treatments achieved an abundance ~80%, the identity of the dominant genotype differed. For example, the mean relative abundance of the dominant genotype in the warmed treatment was 82.9% and that same genotype's abundance dropped to 44.5% in the clipped treatment (Figure S3a). The modest initial differences in shoot length and rhizome volume between genets within a pair had no effect on final below-ground biomass (Figure S2).

The ordination of traits allowed us to separate genotypes in trait space based on two principal components, with PC1 explaining 40.4% of the variance and being negatively correlated with maximum electron transport rate (ETR_{max}) and the above-ground to below-ground biomass ratio, and PC2 (33% of the variance) positively correlated with photosynthetic efficiency (alpha; Table S2). The mean multivariate trait composition of winning genotypes (relative abundance > 0.7) varied among treatments, as shown by the clear separation of control, warmed only and clipped treatments in trait space (Figure 3a). Winning genotypes in the clipping treatments had lower values of maximum electron transport rate (ETR_{max}) and above-ground to below-ground biomass ratio, while warming favoured lower photosynthetic efficiency (alpha) relative to the control. Within each treatment, the winning (relative abundance > 0.7), coexisting (relative abundance between 0.3 and 0.7) and losing (relative abundance < 0.3) genotypes also separated across trait space (Figure 3b). In non-clipping treatments, winning genotypes had higher maximum electron transport rate (ETR_{max}) and above-ground to below-ground ratio values than the remaining abundance categories, whereas in clipping treatments winning and losing genotypes shared trait space but had higher photosynthetic efficiency (alpha) values relative to the coexisting genotypes.

Genotypic evenness was higher in control and warming + clipping treatments relative to warming or clipping only treatments, as shown by an interaction between warming and clipping on evenness ($F = 3.6$, $p = 0.07$, Figure 4).

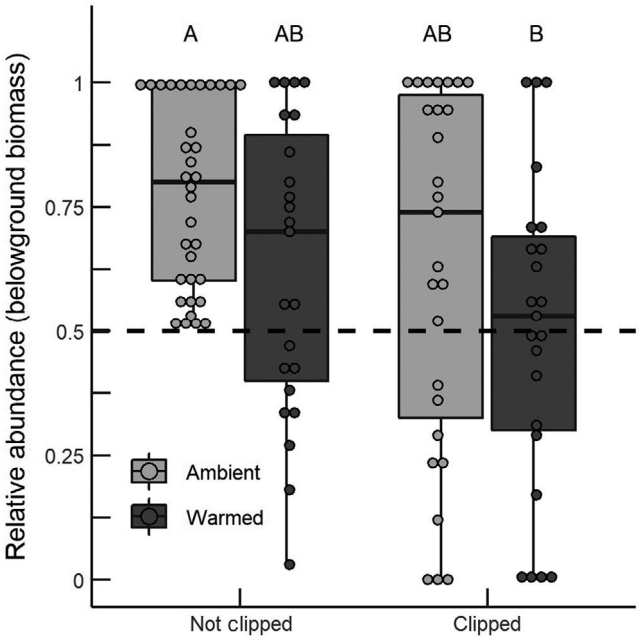

FIGURE 2 Relative abundance of genotype that was dominant (i.e. highest below-ground biomass between pairs of *Zostera marina* genotypes) in the unmanipulated control across each of the warming and clipping treatment combinations. Relative abundance below 0.5 indicates that the identity of the dominant genotype changed relative to the control. Data are visualized using boxplots where the dark horizontal line shows the median and each dot represents an individual replicate. Light grey colour: ambient temperature treatment; dark grey colour: warmed temperature treatment. See Figure S3 for figures of the relative abundance of the dominant genotype referenced to each of the remaining treatments. Letters show results of post-hoc pairwise permutation tests corrected for multiple comparisons: treatments with the same letter were not distinguishable

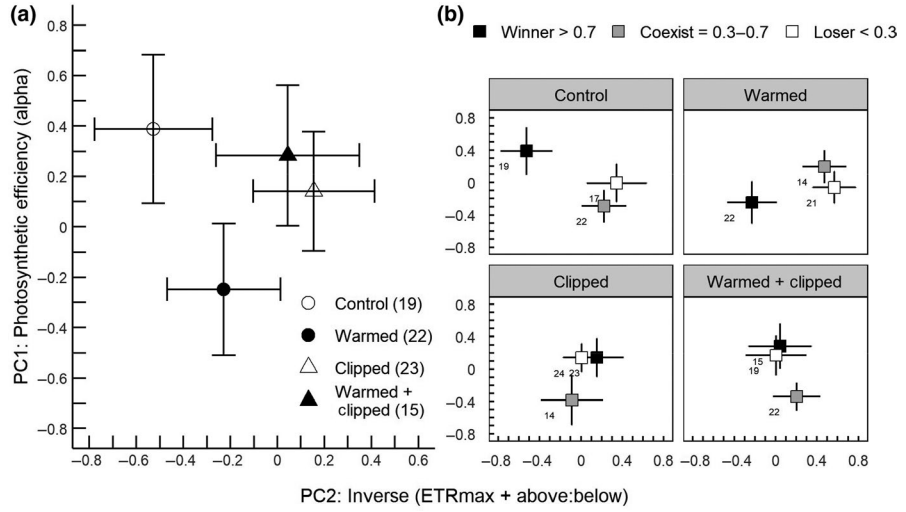

FIGURE 3 The association of traits and interaction outcome between genotypes of *Zostera marina*. (a) Mean multivariate trait index of the 'winning' genotypes (i.e. genotype with a relative abundance of its below-ground biomass > 0.7 within the pair) across experimental disturbance regimes. Open circles: control; closed circles: warmed; open triangles: clipped; closed triangles: warmed + clipped. (b) Mean multivariate trait index of the genotypes within each treatment categorized by their relative abundance. Box colour indicates interaction outcome—black: winning genotypes (relative abundance > 0.7); grey: both genotypes coexist (relative abundance between 0.3 and 0.7); white: losing genotypes (relative abundance < 0.3). The multivariate trait index was extracted from a principal component analysis of the following traits: photosynthetic efficiency (alpha), maximum electron transport rate (ETR_{max}) and above-ground to below-ground resource allocation (the above:below biomass ratio; see Table S2 for PCA details). For both (a, b), *x*- and *y*-error bars represent standard error of the mean, numbers indicate sample size, and the *x*-axis is drawn such that low values of PC2 represent high values of maximum electron transport rate (ETR_{max}) and the above-ground to below-ground biomass ratio

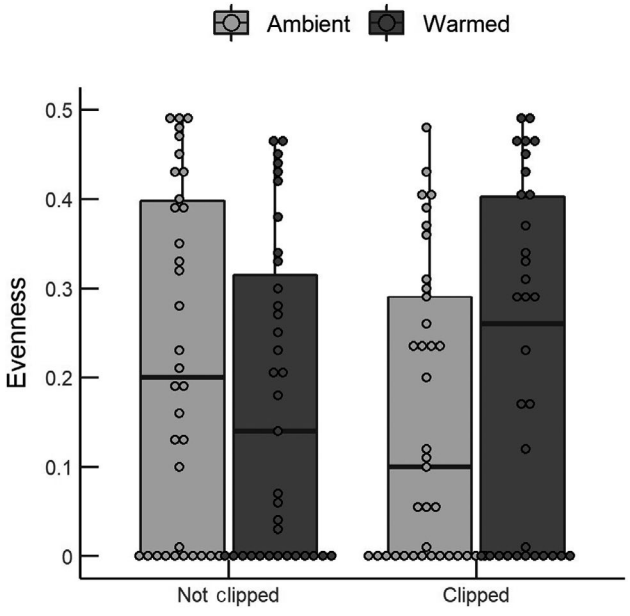

FIGURE 4 The effects of a warming event and clipping disturbance on evenness within randomly paired genotypes of *Zostera marina*. Light grey colour: ambient temperature treatment; dark grey colour: warmed temperature treatment. We calculated evenness as the relative abundance of the genotype with the lower below-ground biomass; a value of 0.5 indicates complete evenness and 0 indicates exclusion of one genet within the pair. Data are visualized using boxplots where the dark horizontal line shows the median and each dot represents an individual replicate. Warming × clipping interaction: $F = 3.6$, $p = 0.07$

4 | DISCUSSION

Our results demonstrate how two distinct seasonal disturbances can alter the biomass, genotypic composition and evenness of a clonal plant assemblage. Warming and clipping each reduced at least one metric of eelgrass abundance. However, the two disturbances acted synergistically to reduce below-ground biomass, suggesting that fall warming may reduce the ability of plants to tolerate winter grazing (Figure 1; Table 1). We also showed that the identity of the dominant genotype within a pair often shifted among the four disturbance treatments (Figure 2). The traits of the winning genotypes differed across the treatments (Figure 3a), allowing us to develop hypotheses about the mechanisms underlying these shifts. In the absence of disturbance and in the warming only treatments, the winning genotypes were phenotypically distinct from the subordinate clone (Figure 3b). However, within the clipping treatments (both clipping only and warming + clipping), the winning and losing genotypes were indistinguishable based on traits (Figure 3b). This suggests that disturbance type influences whether our measured traits drive the outcome of inter-genotypic interactions in *Zostera*. Relative to a single disturbance, we found that sequential disturbances increased evenness among genotypes (Figure 4) by creating variable selection that favours different genotypes in different seasons, equalizing the relative abundance of competitors (conceptualized in Figure 5).

Interactions between sequential disturbances can occur when one disturbance alters the response to a second disturbance (Buma, 2015; Paine et al., 1998; Turner, 2010). Here, we showed that

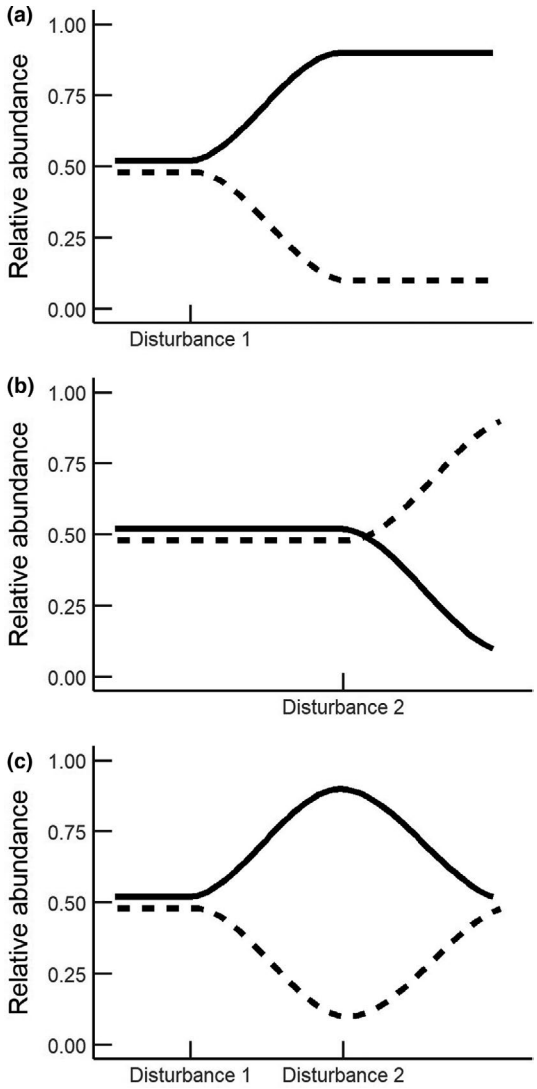

FIGURE 5 Conceptual model of how sequential disturbances maintain genotypic diversity via fluctuating selection. (a, b) Assemblages that only experience one disturbance type would be dominated by the genotype with traits best suited to the environmental conditions created by that disturbance type, leading to unequal abundance of the two genotypes. (c) However, temporally separated disturbances that create environmental conditions that favour different genotypes can maintain an even relative abundance over time. Solid and dashed lines represent genotypes with distinct trait combinations

elevated temperatures in the fall can intensify the damaging effects of grazing disturbance in the winter. Consistent with previous findings, warming and clipping independently reduced the density and biomass of *Zostera* assemblages (*warming*: DuBois et al., 2020; Kim et al., 2020; Moreno-Marín et al., 2018; Reynolds et al., 2016; *clipping*: Hughes, 2006; N.M. Kollars & J.J. Stachowicz, unpubl. data; Ruesink et al., 2012), but these reductions were more severe when the assemblages experienced both disturbance types (Figure 1, Table 1).

Interactions between warming and grazing are common in plant systems but are often complicated by factors such as selective grazing (e.g. Post & Pedersen, 2008) and water availability (e.g. Carlyle et al., 2014). However, our personal observations indicate that the geese do not discriminate among genotypes, and water stress is obviously not a contributing factor in aquatic systems. The above studies also applied the two disturbance types simultaneously rather than sequentially, which likely further differentiates the underlying mechanism driving the interaction in our study relative to other experiments. We hypothesize that warming and clipping interact such that warming reduces the plant's tolerance to clipping via physiological effects on below-ground (storage) resources. Warming often causes plants to increase allocation to above-ground production at the cost of below-ground biomass to compensate for increased respiration rates (in seagrass: Clausen et al., 2014; Dubois et al., 2020; reviewed for plants more generally in Lin et al., 2010). Consequently, warming of *Zostera* reduces rhizome elongation (Reynolds et al., 2016) and carbon storage (Moreno-Marín et al., 2018) and warming events that immediately precede winter light limitation may intensify these reductions (Moreno-Marín et al., 2018). Given that the ability for plants to recover from above-ground herbivory generally depends on below-ground storage (reviewed in Thomas et al., 2017), especially during light limitation in the winter months (Alcoverro et al., 1999; Govers et al., 2015), increased above-ground to below-ground biomass ratios as an adaptive response to warming may be maladaptive if the plants are subsequently grazed, leading to a synergistic negative effect of the two disturbances. Thus, trade-offs may exist such that genotypes resilient to warming are also more vulnerable to grazing. If true, the warming and clipping treatments might select for different genotypes and the combination of the two disturbances could ultimately mitigate competitive exclusion (conceptualized in Figure 5).

Indeed, we did find that the dominant genotype within a pair often changed under the different environmental regimes and a single genotype rarely dominated across all four treatment conditions (Figure 2; Figure S3). The no disturbance treatment favoured genotypes with trait values related to enhance above-ground processes (i.e. photosynthetic performance and greater allocation to the production of shoot tissue). Genotypes with these traits likely have the competitive advantage when the only stress the assemblage experiences is light limitation from the combined effects of self-shading and winter light reductions (e.g. DuBois et al., 2019). As expected, winners in the warmed treatment also had traits consistent with higher allocation to above-ground production (Clausen et al., 2014; DuBois et al., 2020). However, winners in clipped treatments had a lower above-ground to below-ground biomass ratio (higher values of PC1) than winners in unclipped treatments, supporting the idea that above-ground biomass removal selects for genotypes that preferentially allocate resources to below-ground tissues (storage). Clipping also appeared to have stronger selective effects than warming when the two disturbances happened sequentially, as favoured genotypes with the lower PC1 and PC2 values after warming no longer dominated the assemblage in the warming + clipping treatment. This latter result is consistent with the much larger effect of clipping on biomass than warming (Figure 1) but may also result because clipping happened closer in time to the measurement of the outcome than warming, which was applied months earlier.

The multivariate trait index differed between winners and losers within a treatment in expected ways in the no disturbance and warming treatments but not in the clipping treatments (Figure 3b). Under both clipping only and the warming + clipping treatment, genotypes with the highest and lowest abundance in a pair overlapped in trait space. The mechanism underlying this result is unclear, but we offer two possibilities. First, our selection of traits may not sufficiently predict the interaction outcome when the plant experiences defoliation. However, our trait index could predict instances of coexistence within pairs under the clipping treatments (Figure 3b), which suggests that metrics of photosynthetic capability and resource allocation at least partially contribute to interaction outcome. A second possibility is that our results indicate that exclusion happens stochastically after clipping, even though our multivariate trait index could predict instances of coexistence (Figure 3b). In contrast to warming events in which biomass loss occurs as a delayed consequence to physiological stress (Reynolds et al., 2016; Figure 1b; Table 1), clipping directly removes the photosynthetic tissue of the entire assemblage, thus reducing both total biomass (Figure 1b) and temporarily alleviating above-ground competition. In this case, stochastic processes may drive interaction outcome because persistence depends more on the ability to recover from biomass loss and less on genotype-specific competitive abilities. Though we cannot definitively identify the precise mechanism, our results do show that warming and clipping affected inter-genotypic interactions via different processes.

These differential effects of the warming and clipping disturbances on the relative abundance and the mean multivariate trait value of the dominant genotype within a pair may explain why we observed an increase in evenness among pairs in the sequential disturbance treatment relative to warming or clipping alone (Figure 4). One possibility is that the interaction between warming and clipping on biomass loss (Figure 1b; Table 1) contributed to the increase in evenness. This would occur if biomass reduction was severe enough to temporally limit the importance of competitive interactions between genotypes and consequently delay exclusion (Chesson & Huntly, 1997). While this may play a role, biomass reduction alone cannot explain the increase in evenness in the warming + clipping treatment because the clipping only treatment also reduced biomass relative to warming only but did not affect evenness (see Section 3). Instead, we argue, as above, that the two disturbance types antagonistically favour genotypes with different traits such that the subsequent application of both disturbances can negate the trade-off and promote evenness within the assemblage.

The variation we observed in interaction outcome in our laboratory mesocosm suggests that fluctuating environmental conditions maintain the genotypic diversity of *Zostera* in the field. Though our experimental design only isolated interactions between two genotypes at a small spatial scale, a mesocosm-based experiment allowed us to explicitly test the effects of varying disturbance regimes on the fine-scale interactions between ramets of distinct genets. In the field, *Zostera* meadows can be highly diverse (Hughes & Stachowicz, 2009; Kamel et al., 2012; Olsen et al., 2004) and shoots of different genotypes grow intertwined at spatial scales analogous to the size of the pots we used in the mesocosm (N.M. Kollars & J.J. Stachowicz, unpubl. data). Consequently, our design of randomly pairing unique genotypes may represent the types of genotypic interactions that occur in natural assemblages. While our experimental design cannot explicitly test how an individual genotype responds to sequential disturbances, our results do indicate that universal 'winners' are rare, as the four treatment regimens favoured genotypes with different trait combinations (Figure 3). Furthermore, we only tested the effects of two disturbance types on interaction outcome. In addition to warming and grazing, seasonally variable above-ground biomass loss may result from pulses of freshwater inundation, tidal-dependent desiccation stress, shading by algal blooms, and outbreaks of disease or invasive species (reviewed in Orth et al., 2006; Short & Wyllie-Echeverria, 1995). It seems likely that these additional stresses might favour genotypes with different trait combinations.

A sufficient frequency of shifting environmental conditions would perpetually prevent competitive exclusion and thus contribute to the maintenance of genotypic diversity in a similar way to how fluctuating environments are thought to maintain species diversity in other systems (conceptualized in Figure 5). Previous studies have attributed the maintenance of within-species diversity to environmental heterogeneity (e.g. Carvalho & Crisp, 1987; Chang & Smith, 2014; Ellstrand & Roose, 1987; Grosberg, 1988; Steiner & Nowicki, 2019), but our study expands the context in which disturbance can influence diversity by showing that fluctuating selection may result from the sequential application of different disturbance types and not simply seasonal changes in a single agent. Given the widespread evidence for the role of environmental heterogeneity in driving diversity maintenance at the community (among species) level (e.g. Connell & Slatyer, 1977; Hutchinson, 1961; Miller & Chesson, 2009), this principle may hold across levels of biological organization. Finally, our results suggest that sequential disturbances play an especially important role in the maintenance of diversity when each disturbance type selects for genotypes with traits that are disfavoured by the other disturbance type, promoting coexistence among genotypes.

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AUTHORS' CONTRIBUTIONS

N.M.K., K.D. and J.J.S. conceived the study; N.M.K. and K.D. performed the experiment; N.M.K. analysed the data and wrote the manuscript with substantial input from all authors.

DATA AVAILABILITY STATEMENT

Data and R code are available at Zenodo.org as the 'CookedGoose' project https://doi.org/10.5281/zenodo.4041613.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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