Context-dependent resilience of intertidal seagrass and venerid clams after hyposalinity stress

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ABSTRACT: Reduced salinity in estuarine areas can negatively affect intertidal seagrass meadows where clams are harvested. However, legacy effects of hyposalinity on seagrasses and infaunal clams and on their interactions have seldom been studied. Legacy effects were examined in the intertidal seagrass *Zostera noltei* and juveniles of the clams *Venerupis corrugata*, *Ruditapes decussatus* and *R. philippinarum*. Low-salinity stress was applied to mesocosms that included assemblages of seagrass and the 3 clam species that were subsequently transplanted to 2 shellfish beds characterized by different environmental conditions (i.e. seawater temperature, salinity and nutrient concentration). After 2 mo, the morphological and biochemical traits of *Z. noltei* and the growth and mortality of the clams were measured. Past hyposalinity stress increased clam mortality and decreased *Z. noltei* leaf length. The native *V. corrugata* was the most vulnerable to past hyposalinity, whereas the introduced clam *R. philippinarum* was the most resilient. The presence of clams was associated with greater nitrogen content and biomass of the above-ground parts of *Z. noltei*. Survival of the clams after past hyposalinity stress was greater below *Z. noltei* than below bare sediment, indicating persistent positive interactions following the harsh environmental conditions and during low-salinity periods in the field. At the colder and more nutrient-rich site, positive interactions were more frequent and *Z. noltei* performed better. This supported the influence of abiotic conditions on the recovery process. Nonetheless, enhanced survival of clams below *Z. noltei* indicates that *Z. noltei* could favour the sustainability of these shellfisheries after exposure to extreme rainfall events.

KEY WORDS: Shellfisheries · Climate change · Low-salinity stress · *Ruditapes philippinarum* · *Ruditapes decussatus* · *Venerupis corrugata* · Seagrass · Recovery

1. INTRODUCTION

Estuarine areas, and especially intertidal communities, are subjected to variations in temperature and humidity in synchrony with the flowing and ebbing tides, which exposes estuarine organisms to heating and desiccation (Rafaelli & Hawkins 1996). Moreover, these areas are prone to frequent salinity fluctuations due to freshwater discharges from rivers, which can lead to low-salinity events (Gillanders & Kingsford

2002, Arnell & Gosling 2016, Des et al. 2021). These salinity fluctuations can range from absolute values above 30 to values near 0 during periods of heavy rain in the estuaries of northwest (NW) Iberia (Parada et al. 2012). Climatic stressors can be particularly harmful to intertidal communities since they can worsen the effect of background stressors already present in estuaries. These climatic stressors include increased temperatures, rising sea levels, acidification and increased frequency of extreme events such as heatwaves, floods and heavy rainfall (Arnell & Gosling 2016, IPCC 2021).

In soft-bottom intertidal communities, clams of the species *Venerupis corrugata* (Gmelin, 1791), *Ruditapes decussatus* (Linnaeus, 1758) and *R. philippinarum* (A. Adams & Reeve, 1850) are commercially important species in northern Africa, Asia and southern Europe. The non-native *R. philippinarum* is now the major contributor to clam landings in Europe, whereas the productivity of the native species is decreasing (www.fao.org, www.sealifebase.ca). Shellfishing beds with these clam species frequently coexist with meadows of small intertidal seagrasses of the genus *Zostera* (Park et al. 2011, Li et al. 2017), with *Z. noltei* Hornemann predominating in intertidal banks in southern Europe (Guimarães et al. 2012, Garmendia et al. 2021).

All 3 of the aforementioned clam species are often negatively affected by low-salinity events (Parada et al. 2012). Clams respond to decreased salinity by closing their valves to maintain osmotic equilibrium and by reducing feeding and respiration (Gosling 2015). Low-salinity events also have critical effects on burrowing (Haider et al. 2018), reproduction (Vázquez et al. 2021), scope for growth (Domínguez et al. 2020) and survival of adult clams (Carregosa et al. 2014a, Rato et al. 2022). The mortality of these species in shellfish beds in NW Spain is thus likely to increase in the period 2070–2099 due to the predicted increased frequency of low-salinity events (i.e. under Representative Concentration Pathway 8.5; Des et al. 2021. Note that for this region, some model predictions are for decreases in rainfall events; see Cardoso-Pereira et al. 2020, Cos et al. 2022). Sublethal and lethal effects of low-salinity events are potentially greater in juvenile clams (Rato et al. 2022) and cockles (Peteiro et al. 2018) than in adults, with negative consequences for the maintenance of reproductive stocks. However, studies of these effects are scarce. Plants of the genus *Zostera* are euryhaline but display a wide range of responses to reduced salinity, with reports of positive (Short 1999), neutral (Cardoso et al. 2008, Sousa et al. 2017) and negative effects (Char-

pentier et al. 2005, Nejrup & Pedersen 2008, Solá et al. 2020).

Depending on the intensity, frequency and duration of these events, the growth, development and survival of macrophytes and intertidal sessile or low-mobility invertebrates can be altered (Gillanders & Kingsford 2002, Domínguez et al. 2020, Woodin et al. 2020, Vázquez et al. 2021). Consequently, changes in the structure, functioning and distribution of communities can occur (Mieszkowska 2009). The sublethal effects of stressors may persist long after the stress ends, leading to reduced fitness and additional mortality after some months. For instance, legacy effects of environmental stressors were reported in copepod larvae under low salinity (Richmond et al. 2007), in macroalgae assemblages under elevated temperature and pCO_2 (Vaz-Pinto et al. 2013) and in mussels under elevated temperatures and decreased pH (Gestoso et al. 2015). By contrast, negative effects of acidification on shell density of the clam *Mercenaria mercenaria* (Linnaeus, 1758) decreased after several weeks of grow-out recovery, with varying functional responses depending on the environmental context (Grear et al. 2020).

However, several positive interactions between intertidal seagrasses and commercial clams may reduce the potential legacy effects of past environmental stress (Peterson & Heck 2001, Herrera et al. 2022). In this sense, some clam–seagrass interactions can be classified as facilitative and are thus important for community stability. Facilitation occurs when one species benefits from the other (enhancement of growth, survival, reproduction) due to its alteration of the environment, provision of resources or protection from biotic or abiotic stress (Bronstein 2009). Such positive interactions may be stronger under harsher than under milder stress conditions, consistent with community ecology theory (Bertness & Callaway 1994), as long as the performance and/or the survival of the organisms driving the positive effect have not been critically altered by the stress. For instance, under warming conditions, juveniles of *R. decussatus* living below *Z. noltei* were found to grow at faster rates than those living below bare sediment (Román et al. 2022). Likewise, the recovery of *Z. marina* and *Halodule wrightii* Ascherson scarred by boat propellers was enhanced in the presence of *M. mercenaria* (Donaher et al. 2021).

Environmental variables such as irradiance and temperature can influence the performance of organisms and the direction and magnitude of their interactions. For example, the bivalve-facilitated recovery of seagrass after physical disturbance was enhanced during

the growing season (Donaher et al. 2021), and the positive reciprocal seagrass—bivalve interactions generally increased with increasing water temperatures in subtidal, but not intertidal, habitats (Gagnon et al. 2020). Site-specific variables such as temperature, nutrient levels and salinity can condition the long-term effects of past salinity stress, and the resilience of organisms may then improve under less extreme conditions. Therefore, the present study emphasizes the importance of considering the context-dependency in the recovery trajectories of coastal systems.

To our knowledge, the reciprocal interactions between commercial juvenile clams and *Z. noltei* after past low-salinity stress have not been tested. A better understanding of these interactions is essential, as climate change has numerous social and economic effects on these shellfisheries (Villasante et al. 2022). Nonetheless, the prevalence of positive interactions can enhance the sustainability of shellfish resources and seagrass ecosystems under global change scenarios, with positive ecological and social implications. Furthermore, under the recently increased efforts focused on coastal rewilding, knowing how these systems will respond to future events of hyposalinity can help in better predicting the success of seagrass restoration initiatives.

The aim of this study was to evaluate the legacy effects of low-salinity stress induced in a mesocosm experiment, with subsequent transplantation of juveniles of the 3 commercially important clam species (*V. corrugata*, *R. decussatus* and *R. philippinarum*) and the intertidal seagrass *Z. noltei* to the field. The main hypotheses tested were (1) exposure to low salinity has legacy effects on the growth and biochemistry of *Z. noltei* and on the survival and growth of the 3 clam species, (2) the reciprocal positive interactions between *Z. noltei* and clams reduce the negative legacy effects of past low salinity, (3) the resilience of clams after past hyposalinity stress varies between species, and (4) the resilience of clams and *Z. noltei* is enhanced under conditions of lower temperature and higher nutrient availability.

2. MATERIALS AND METHODS

2.1. Salinity stress in mesocosms

Approximately 100 m^3 of sediment was collected on 8 and 9 April 2021 from the Combarro shellfish bed, Ría de Pontevedra, NW Spain (42° 26' 12.4'' N, 8° 41' 25.3'' W) and sieved through a 5 mm mesh. In a *Zostera noltei* canopy (mean shoot density: 4892 ±

1255 shoots m^{-2}, n = 4), *Z. noltei* plants were collected as complete units comprising the whole plants and the sediment surrounding the rhizomes (approximately 0.4 m^3, covering an area of 4 m^2 and digging to 10 cm depth), with care being taken to avoid breaking the plants. The sediment and plant samples were transported to the mesocosm facilities of the Toralla Marine Science Station (ECIMAT, Universidade de Vigo; https://cim.uvigo.gal/), located in the same region around 50 km from the donor site, where 108 experimental units were established.

The units consisted of perforated plastic baskets (height: 15 cm; length × width: 23.6 × 23.6 cm, approximately 557 cm^2), which were lined with thick gardening mesh to prevent sediment loss while allowing water circulation. The baskets were filled with sediment from the shellfish bed (Fig. 1). The seagrass samples were placed on top of the sediment in two-thirds of the baskets (n = 72), and the other one-third of the baskets (n = 36) were left with bare sediment. The bare sediment or *Zostera* was level with the top of the basket. Nine baskets (6 with *Zostera* and 3 with bare sediment) were haphazardly placed into each of 12 mesocosm tanks, each with a capacity of 800 l (height × width × length: 60 × 102 × 133 cm) and supplied with running filtered (50 µm) seawater at ambient temperature. The baskets were held in the tanks for 32 d before the beginning of the experiment to allow the sediment to stabilize and seagrass to take root. Over the acclimation period in the mesocosm, the salinity was 34.71 ± 0.72 psu, the seawater temperature was 15.33 ± 0.85°C and the global radiation was 506.38 ± 166.46 W m^{-2} (mean ± SD, n = 2012). These values were similar to those at the donor site during the same period (salinity: 34.54 ± 0.90; temperature: 15.59 ± 1.24°C; global radiation: 430.04 ± 226.55 W m^{-2}; n = 73; www.intecmar.gal/Ctd/). Approximately 800 juvenile clams of each species (*R. decussatus*, *R. philippinarum* and *V. corrugata*) (size between 15 and 20 mm) obtained from seed stock were submerged for 2 h in a solution of ambient seawater with the fluorochrome marker calcein (250 mg l^{-1}, Sigma, CAS 1461-15-0) to mark the border of the clam shells. Immediately after exposure to calcein, 11 clams of each species (33 clams per basket) were seeded into 6 baskets (3 with *Z. noltei* and 3 with bare sediment) in each tank, and 3 baskets with *Z. noltei* were left without clams. Clam densities were equivalent to 592 ind. m^{-2}, which is within the range of juvenile seeding densities in shellfish beds in Spain (300—800 ind. m^{-2}; Navajas et al. 2003). The clams were left to burrow and acclimate in the sediment for 24 h.
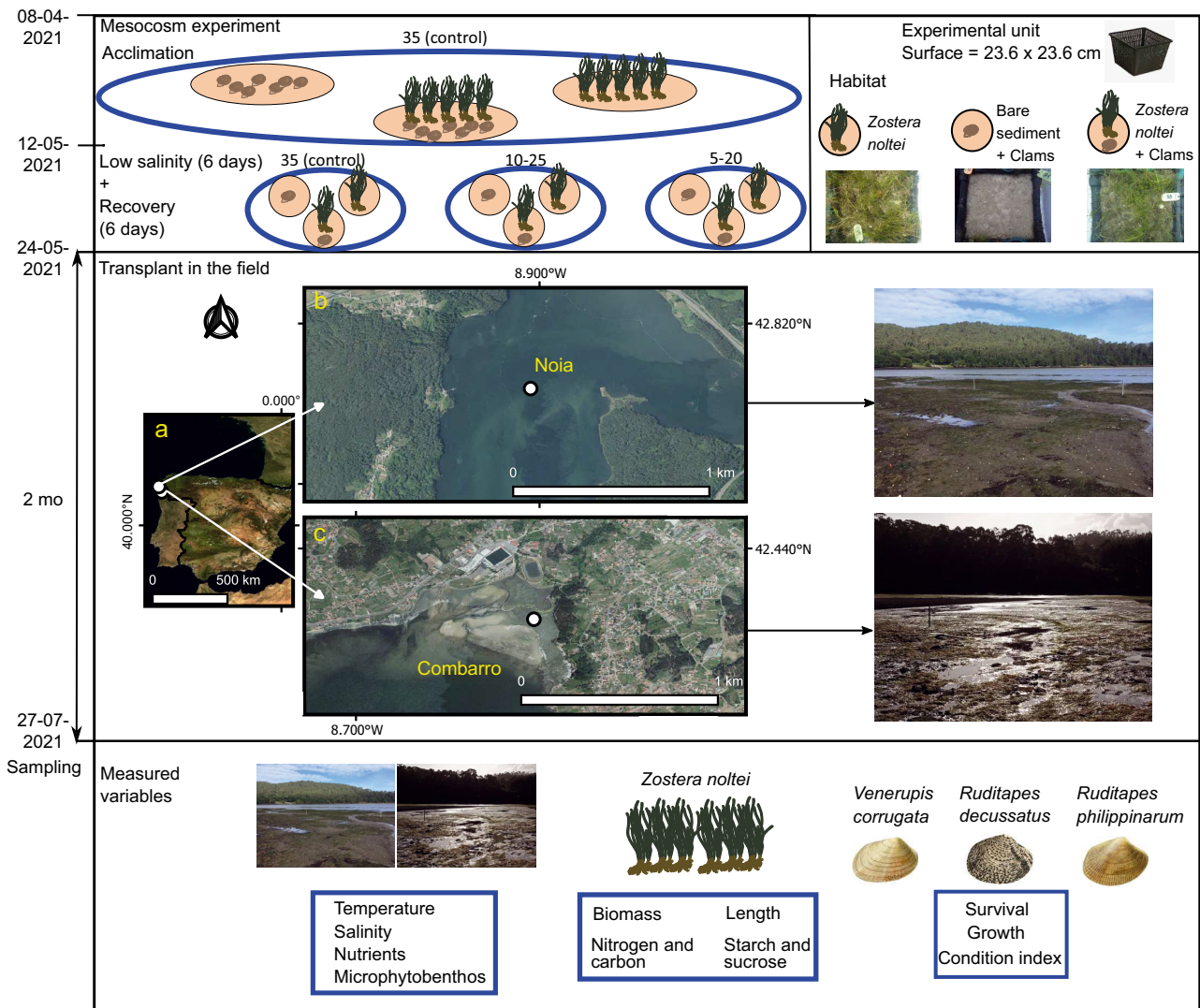
Fig. 1. Experimental scheme showing (a) location of the transplant sites on the coast of NW Iberian Peninsula and detail of (b) the relatively continuous seagrass meadow at Noia site (Ria de Muros-Noia) and (c) the mostly patchy seagrass meadow at Combarro site (Ria de Pontevedra)

An automatic semi-diurnal tidal regime emulating conditions in the shellfish beds that have sublethal but not lethal effects on adult clams (Domínguez et al. 2020) was applied. Thus, the salinity decreased as the tide was going out and increased as the tide was coming in. Four replicate tanks were subjected to each of the 3 salinity treatments for 6 d: salinity of 20 at high tide and 5 at low tide; salinity of 25 at high tide and 10 at low tide and salinity of 35 throughout (control) (salinities hereafter referred to as 5, 10 and 35, respectively).

Natural photosynthetically active radiation between 10:00 and 20:00 h at the mesocosms during the application of treatments averaged 1145.78 ± 562.41 μmol photon m^{-2} s^{-1} (mean ± SD). The clams were fed daily with a mixture of *Isochrysis galbana* (TISO), *Tetraselmis suecica*, *Chaetoceros gracilis* and *Rodomonas lens* (1% dry weight [DW] of microalgae per DW clam). Dead clams on the sediment surface were counted and removed daily during the salinity treatments in the mesocosm. After the salinity stress treatment, all of the tanks were maintained at a constant salinity of 35 for 6 d, and the prepared baskets were then transplanted to the field. A simultaneous experiment was conducted in the same mesocosms, where the same treatments were applied to other baskets and response variables of clams and *Z. noltei* were evaluated at the end of salinity stress in the laboratory (Román et al. 2023).

2.2. Transplantation in the field

The transplant sites were intertidal flats characterized by different temperatures and pluviometry (Table S1 in the Supplement at www.int-res.com/articles/suppl/m729p117_supp.pdf), located in NW Spain. One site was in the inner Ría de Muros-Noia (hereafter Noia; 42.81594° N, 8.90054° W), located 75 km from the donor site and 120 km from the mesocosm, respectively. The other transplant site was in the Ria de Pontevedra (hereafter Combarro; 42.43564° N, 8.68893° W), which was also the donor site. The experimental setup included Past salinity (35, 10 and 5), Habitat (*Z. noltei*, bare sediment + clams and *Z. noltei* + clams) and Site (Noia, Combarro) as fixed orthogonal factors (n = 6). The baskets were covered with plastic netting with 2.8 cm diagonal openings to reduce access of large predators in the field. Half of the baskets were transferred to the Combarro shellfish bed on 24 May 2021, and the other half of the baskets were transferred to the Noia shellfish bed on 26 May (Fig. 1). At each site, the baskets with *Z. noltei* were haphazardly placed within a *Z. noltei* meadow and baskets with bare sediment were placed within bare sediment areas. All baskets were buried so that the upper surface was level with the surrounding sediment, and they were then left for 2 mo.

The baskets were retrieved on 23 July in Combarro and 26 July in Noia. The sediment in each basket was sieved *in situ* and the clams and seagrass samples were transferred to the laboratory and frozen at −20°C.

The temperature in the sediment (3 and 8 cm depth) at the transplant sites was recorded every 30 min with EnvLoggers placed on PVC sticks buried vertically in the sediment (Electric Blue CRL, Vairão; EnvLogger v.2.4) (n = 2). Immersion and emersion temperatures were measured every 30 min with mini-CTDs (Sea-Star, DST Logic CTD v.8.17, Star-Oddi) placed 10 cm above the sediment surface. Unfortunately, the CTD in the Noia site was lost after 1 mo. The weekly concentrations of nitrate, nitrite, ammonium and phosphate in surface waters (0−5 m depth) at both sites during the transplant were extracted (using WebPlotDigitizer) from data published by the Technological Institute for the Marine Control in Galicia (Intecmar; www.intecmar.gal). Hourly salinity data for both sites were obtained from the MeteoGalicia MOHID Water Modelling System ocean forecasts (https://mandeo.meteogalicia.es/thredds/catalogos/MOHID/catalog.html), using the closest valid model pixels (42.78893° N, 8.925092° W at Noia; 42.43063° N, 8.69403° W at Combarro) to the datalogger sites. The

abundance of microphytobenthos (diatoms, cyanobacteria and green algae) in bare sediment and sediment below *Z. noltei* at upper, mid and low intertidal levels of each site was measured by chlorophyll *a* fluorescence, with a BenthoTorch device (bbe Moldaenke) (n = 10).

2.3. *Zostera noltei* traits

The length of *Z. noltei* was measured before and after the 2 mo periods in the field. For this purpose, a square of 10 × 10 cm was delimited in each basket by tying cable ties to the plastic mesh, and the length of the longest leaf was measured in a maximum of 20 shoots. The C and N content was determined in 576 samples of *Z. noltei* leaves. From each basket with *Z. noltei* (72), a maximum of 8 replicates each consisting of 15 shoots (equivalent to 20 mg DW) were dried (60°C, 48 h) and ground to a fine powder for determination of the C and N contents in a Fisons Carlo Erba EA1108 elemental microanalyzer, at the analytical facilities of the University of Vigo (CACTI-UVIGO). Approximately 25 apical rhizomes (equivalent to 0.05 g DW), consisting of the first 2 internodes, were separated in each basket and dried (60°C, 48 h). The samples were then ground for analysis of starch and sucrose contents by resorcinol and anthrone assays, respectively, both standardized to sucrose, according to Olivé et al. (2007). The above- and below-ground biomass (DW, 60°C, 48 h) of *Z. noltei* in all baskets (n = 72) was quantified.

2.4. Survival, growth and condition index of clams

Visible dead clams that were lying on the sediment surface were counted and removed daily during the application of salinity treatments in the mesocosm. The final number of live clams was counted in the baskets retrieved from the field. Dead clams buried in the sediment could not be counted, as this would have required digging the sediment and thus disturbing the seagrass and the sedimentary environment. Total clam survival after the salinity stress in the mesocosm experiment was obtained from the final measurements on the last day of the salinity stress experiment, in order to estimate mortality in the field (Román et al. 2023) (see Section 2.5.1). The number of clams that died after transfer to the field and during the recovery period was thus calculated by subtracting the final number of live clams minus the estimated live clams before transplantation.

Shell growth was measured in a total of 1257 clams. The periostracum of the right valves was removed by immersion in an H_2O_2 solution (35%) for 24 h. The valves were then rinsed with tap water, dried at room temperature and examined under a Leica MZ125 microscope with a fluorescence filter (Leica SFL 100) at an excitation wavelength of 470 nm to detect the calcein mark. Shell growth was calculated (in triplicate for each valve) as the difference between the calcein mark and the shell edge: one measurement was made in the centre of the shell edge, aligned with the position of the umbo, and one each at 4 mm above and below the first measurement. The measurements were made with Leica Application Suite V4 image analysis software. Those clams without marks were discarded.

The flesh and shells of all the clams retrieved from the field (n = 1539) were oven-dried (60°C, 48 h) and weighed. The condition index (CI) of clams was calculated at the end of the experiment, following Eq. (1) (Walne & Mann 1975):

$$\text{CI (‰)} = \frac{\text{flesh dw}}{\text{shell dw}} \times 1000 \qquad (1)$$

2.5. Data treatment and statistical analysis

2.5.1. Biological data processing

The shell growth and flesh DW of clams after the field exposure reflected the cumulative response to salinity conditions during both the mesocosm and transplant experiments. Responses immediately after salinity mesocosm treatments could not be obtained directly by measuring the individuals in the baskets transplanted in the field. Therefore, as with the dead clams, these data were extracted from the final measurements on the last day of the mesocosm salinity stress experiment (Román et al. 2023).

The actual number of dead clams was regressed against the number of visible dead clams per basket and species determined in the salinity stress experiment. The equations thus obtained (Table S2) enabled estimation of the number of dead clams per basket after the salinity stress (before transplantation). The median predicted actual number of dead clams per species after salinity stress in each situation (combination of habitat and salinity) was calculated, and robust standard deviations were obtained using the 'estimateSd' function of the 'jointseg' package (Table S3) (Pierre-Jean et al. 2019). The estimated number of live clams per basket and species before transplantation in each situation was calculated using Eq. (2):

$$N_{\text{live clams before transplant}} =$$
$$11 - \text{median predicted true dead clams} \qquad (2)$$

A data set with a binary response (0 = dead; 1 = alive) was created for the survival of clams after transplantation, where the total number of rows per basket corresponded to $N_{\text{live clams before transplant}}$ obtained in Eq. (2).

The shell growth and flesh DW of live clams per situation after the salinity stress experiment were estimated from the final measurements on the final day of the salinity stress experiment (Román et al. 2023) through bootstrapping, with 2000 resamplings and 95% confidence intervals. To determine the increase in shell growth and flesh DW in the field, the estimated shell growth and DW after the salinity stress experiment were subtracted from each observation in the transplant experiment.

2.5.2. Environmental data processing

The CTD and MOHID data were separated into emersion and immersion periods (Text S1). To complete the missing seawater temperatures in the Noia site between 26 June and 26 July 2021, the CTD data from Noia were regressed against the temperatures from MOHID between 2 January 2020 and 23 August 2020. The regression ($T_{\text{CTD}} = -1.97$ (± 0.18) $+ 1.15$ (± 0.01) T_{MOHID}; $R^2 = 0.76$, df = 3074, p < 0.001) (Fig. S1) was used to estimate the immersion CTD temperatures for the missing data.

The degree days (DDs) at 3 cm, which represented the amount of heating experienced by the shallow clams like *R. philippinarum*, and at 8 cm, experienced by the deeper clams like *R. decussatus* and *V. corrugata*, were calculated as the sum of positive differences between mean hourly temperatures and the threshold of 20°C, the optimal temperature for performance of adult clams (Domínguez et al. 2021), divided by 24 (no. of hours per day). Salinity unit days (SUDs), analogous to DDs, were calculated as the sum of positive differences between thresholds of 20 and 10 and mean hourly salinities, divided by 24. SUD values were used to represent the cumulative exposure to low salinities during the initial mesocosm treatments and the subsequent recovery period in field sites. SUDs during the mesocosm treatments were derived from salinity sensors in each of the treatment tanks, whereas SUDs during the recovery period were derived from MeteoGalicia MOHID salinity model during the period of the day when the transplant sites were submerged according to the MOHID water level model.

2.5.3. Models

The differences between sites in the 90th percentile of daily temperatures were calculated and subjected to *t*-tests. Between-site differences in daily salinity, weekly nutrients in surface seawater and microphytobenthos abundances were tested using linear models with the 'lm' function in the R package 'stats' (R Core Team 2022).

The effect of the 3 fixed factors, Site (Noia, Combarro), Habitat (*Z. noltei*, bare sediment + clams, *Z. noltei* + clams) and Past salinity (35, 10, 5), on response variables was tested, with Basket included as a random factor, where applicable. The *Z. noltei* response variables were tested using 3-factor models (Site, Habitat and Past salinity), and the clam responses were tested using 2-factor models (Habitat and Past salinity), as different species-specific responses were expected. Furthermore, models for clams were fitted separately for each site because at Combarro at the end of the transplantation, the *V. corrugata* clams previously exposed to a salinity of 5 in the mesocosm were all dead, which precluded fitting full orthogonal models.

Clam survival was fitted using generalized linear mixed-effects models with a binomial distribution of errors and Basket as a random factor using the 'glmer' function from the 'lme4' package (Bates et al. 2015). The models for percent carbon (%C), percent nitrogen (%N) and leaf length of *Z. noltei* and for shell growth and flesh weight increment and CI of clams were fitted using linear mixed-effects models with the 'lme' function in the 'nlme' package (Pinheiro et al. 2022), with Basket as a random factor. Starch and sucrose contents were averaged per basket due to the high variability between replicates. The models for the biomass, starch and sucrose contents of *Z. noltei* were fitted using linear models with no random factor because there was only one observation per basket for these variables.

The full models were simplified using a theoretical approach, by selecting those with the lowest corrected Akaike information criterion (AICc) values. The optimal random structure was first selected (Table S4) and the fixed part of the models was then simplified; those models that did not have any other simpler model nested within were selected (Richards et al. 2011) using the 'dredge' function in the 'MumIn' package (Bartoń 2022) (Tables S5–S9). The final models were subjected to analysis of deviance, through type 3 sum of squares, and the 'Anova' function in the 'car' package (Fox & Weisberg 2019) was used to identify the significant terms.

Prior to performing the statistical tests, outliers for all variables that were 3 times the inter-quartile range above or below the third or first quartiles, respectively, were removed, except in the case of the binary data of clam mortality (modified from Crawley 2013) to increase the confidence of statistical tests without loss of many observations. The percentage of observations lost was below 3%. The normality of the residuals and homogeneity of variances were graphically checked using QQ plots and plots of standardized residuals against fitted values, respectively. When the residuals of the models were not homogeneously distributed, the heteroscedasticity structure for each combination of treatments was defined using the 'VarIdent' function. Effects of fixed factors were considered statistically significant when p-values were below 0.05, whereas for p-values between 0.05 and 0.1, the null hypotheses were accepted but different trends were acknowledged (as almost statistically significant). Post hoc tests were run for the significant terms of the final models. The least-squares means for the significant factors or their combinations were calculated, and pairwise multiple comparisons were carried out using the 'emmeans' function (Lenth 2022). The p-values associated with the post hoc tests were adjusted with Tukey correction to reduce the comparison-wise error rates (Underwood 1997). All data are reported as means ± SE. All statistical tests were conducted using R v.4.2.2. (R Core Team 2022).

3. RESULTS

3.1. Environmental conditions in the transplant sites

Temperatures were higher at the Combarro site than at the Noia site (Fig. 2a–d & Fig. S2). The differences between Combarro and Noia in the 90th percentile of daily temperatures were positive and significant (Table 1). The cumulative DDs above 20°C were greater at Combarro than at Noia at 3 and 8 cm (Table S10). Moreover, salinity was significantly higher at Combarro than at Noia (Table 1, Fig. 2e,f). During the transplant recovery period, SUD values below 20 and 10 revealed a cumulative exposure to low salinity at Noia, but not at Combarro (Table S10).

Ammonium, nitrate and nitrite concentrations in surface waters (μmol l^{-1}) were significantly greater at Noia than Combarro (ammonium: 2.14 ± 0.33 vs. 1.40 ± 0.39; nitrate: 17.21 ± 3.57 vs. 0.42 ± 0.25; nitrite: 0.35 ± 0.03 vs. 0.07 ± 0.01), whereas there were no differences in phosphate concentrations between the
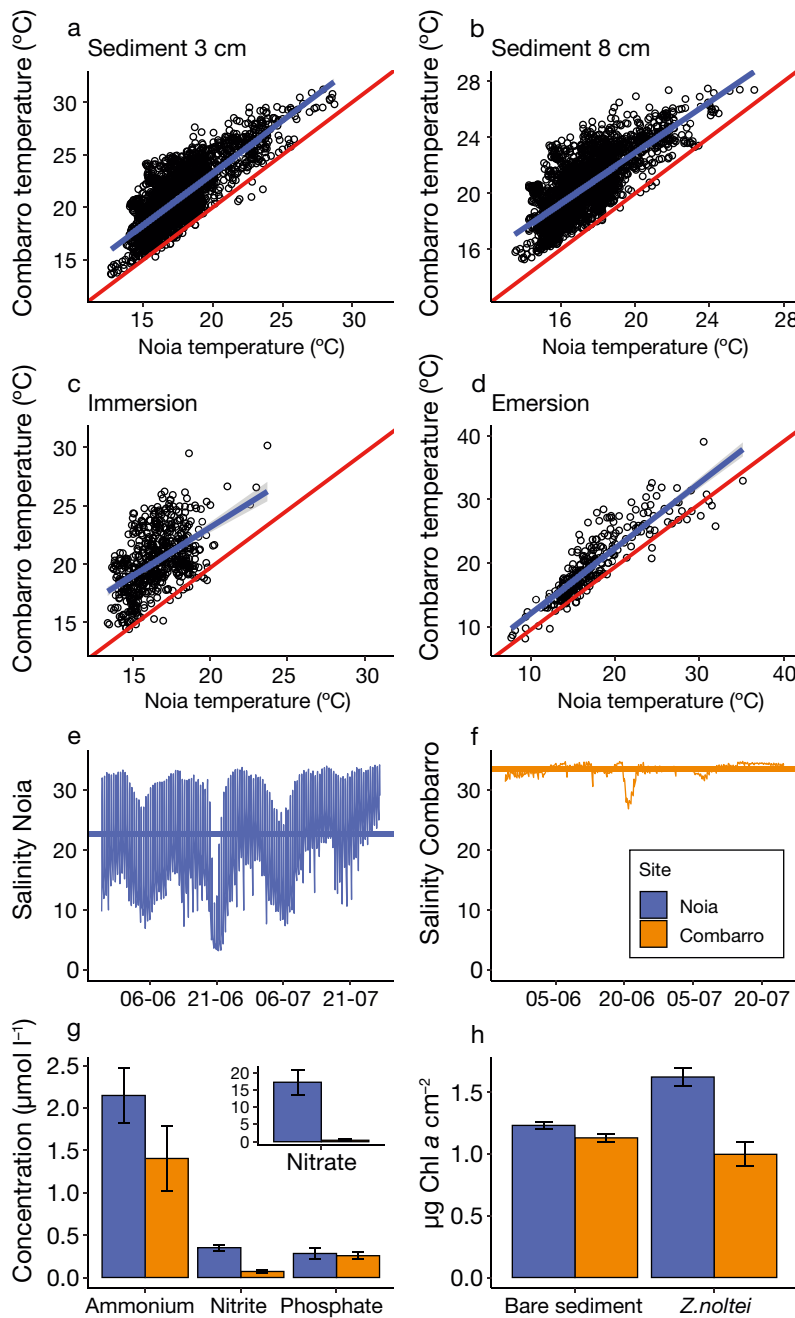
3.2. *Zostera noltei* traits

The leaf length of *Z. noltei* after low-salinity stress in the mesocosm (before transplantation) was not affected by habitat or salinity (Table S5). By contrast, after recovery, the leaves were 29% longer in the plants mixed with clams than in those not mixed with clams at the Combarro site. No such differences were observed at the Noia site, which was characterized by higher nutrient levels. At both sites, *Z. noltei* previously exposed to salinity levels of 10 and 5 had significantly shorter leaves than plants exposed to control conditions at the end of the transplant experiment, although there was a high degree of variability among baskets (Fig. 3a,b, Table 2). The above-ground biomass (DW) of *Z. noltei* was 56% greater in the baskets with clams than in baskets with *Z. noltei* only, independent of site, and the above-ground biomass was 38% greater at Noia than Combarro (Fig. 3c, Table 2). However, the below-ground biomass was 29% greater at Combarro than at Noia (Fig. 3d, Table 2). The C content of leaves did not differ between experimental treatments or sites (Fig. 3e, Table S5). The N content of leaves was 28% greater at Noia than at Combarro (Fig. 3f, Table 2). Habitat also had a significant effect on N concentration, although it depended on past salinity stress (i.e. significant Habitat × Past salinity interaction; Table 2). The N content of leaves was significantly greater in the plants mixed with clams than in those not mixed with clams in the salinity 35 treatment ($t = -3.132$, $p = 0.030$), whereas no differences between habitats occurred in plants previously exposed to the other salinity levels (Fig. 3f, Table 2). The sucrose content of the apical rhizomes did not vary significantly between experimental treatments, whereas the starch

Fig. 2. Environmental variables in the shellfish beds during the transplant experiment. (a—d) Regressions of Combarro temperatures versus Noia temperatures from the *in situ* data loggers. Red lines: the 1:1 line; blue lines: model fits; (e,f) salinity from the MeteoGalicia MOHID model, where horizontal lines indicate the mean values for each study site; (g) nutrient contents in surface water (0—5 m depth) (n = 9) extracted from www.intecmar.gal/Informacion/fito/Evolucion/EvolucionNutrientes.aspx; and (h) chlorophyll *a* in microphytobenthos (n = 30)

sites (0.28 ± 0.06 vs. 0.26 ± 0.04) (Fig. 2g, Table 1). Microphytobenthos (µg cm⁻²) were more abundant in Noia than in Combarro in bare sediment and in the sediment below *Zostera noltei* (Fig. 2h, Table 1).

content was 27% lower in seagrass mixed with clams than in those not mixed with clams at both sites, and it was also significantly greater at Combarro than at Noia (Fig. 3g,h, Table 2).

Table 1. Environmental variables at the transplant sites, showing (a) differences between Combarro and Noia in terms of the 90th percentile of daily temperatures and results of *t*-tests and (b) summary of the results of the linear models used to test the effect of site on daily salinity, weekly nutrient concentrations in surface seawater and abundance of microphytobenthos. Significant effects are highlighted in **bold**

(a) Temperature	Mean (±SE) (Combarro − Noia) (°C)	df	*t*	p
Emersion	2.97 ± 2.01	31	8.36	**< 0.001**
Immersion	4.50 ± 2.04	58	16.91	**< 0.001**
3 cm sediment	3.49 ± 1.32	58	20.33	**< 0.001**
8 cm sediment	3.19 ± 1.09	58	22.49	**< 0.001**
(b) Response variable		df	*F*	p
Salinity		1, 122	287.44	**< 0.001**
Ammonium		1, 14	5.999	**0.028**
Nitrate		1, 17	19.721	**< 0.001**
Nitrite		1, 17	54.501	**< 0.001**
Phosphate		1, 17	0.109	0.745
Microphytobenthos in bare sediment		1, 58	5.23	**0.026**
Microphytobenthos below *Zostera noltei*		1, 58	25.8	**< 0.001**

3.3. Survival, growth and CI of clams

At the Noia site, the survival of *V. corrugata* previously exposed to a salinity of 5 was 74 and 73% lower than that of the clams exposed to salinity levels of 10 and 35, respectively, independent of the presence or absence of *Z. noltei* (Table 3). At the Combarro site, none of the *V. corrugata* previously exposed to a salinity of 5 survived, and the survival of individuals previously exposed to a salinity of 10 was 21% lower than that of individuals exposed to a salinity of 35, independent of the presence or absence of *Z. noltei* (Fig. 4a,d, Table 3).

The survival of *R. decussatus* At the Noia site was not affected by any treatment (Table S6), although it varied widely between baskets (Table 3). In Combarro, *R. decussatus* survival varied with prior exposure to salinity stress, with the effect depending on the presence or absence of *Z. noltei* (the Past salinity × Habitat interaction was almost statistically significant) (Table 3). More clams previously exposed to salinity levels of 10 and 35 died in the presence of *Z. noltei* than in bare sediment. In the bare sediment, survival of clams previously exposed to a salinity of 5 was 58 and 61% lower than in those exposed to salinity levels of 10 and 35, respectively, whereas this was not observed in clams mixed with *Z. noltei* (Fig. 4b—e, Table 3).

The survival of *R. philippinarum* at the Noia site depended on the prior exposure to salinity stress and habitat (Table 3). No effect of past salinity was detected on survival of clams placed with *Z. noltei*, whereas in bare sediment the mortality rate of the clams previously exposed to a salinity of 5 was 37% greater than that of clams exposed to a salinity of 35. By contrast, the survival of *R. philippinarum* in Combarro was not significantly affected by habitat or salinity (Fig. 4c—f, Table 3 & Table S6).

There was no clear homogeneous effect of past salinity or habitat on shell growth, flesh increment or CI of clams, and when significant effects appeared, their magnitudes were small. Although the differences between sites were not formally analyzed because of the high mortality rates, shell growth in *V. corrugata* (increase of 57%) and the increase in tissue DW in the 3 species (increase of 141%) was visibly greater at the Noia site than at the Combarro site (Figs. S3 & S4, Tables S7—S9 & S11).

4. DISCUSSION

4.1. Legacy effects of low salinity on *Zostera noltei* and its interactions with clams

After the field recovery period, prior exposure to salinity stress was found to have no effect on the biomass, C and N contents of leaves and carbohydrate contents of apical rhizomes of *Zostera noltei*. Similarly, Cardoso et al. (2008) found that above- and below-ground biomass were not affected by low salinity, although the results were from a 12 yr time series, encompassing several low-salinity stress episodes. In the present study, the only effect of low salinity was shorter leaf length in plants exposed to salinity levels of 10 and 5 than in plants exposed to salinity of 35, which is consistent with the decreased leaf elongation rates observed in *Z. marina* under conditions of low salinity (Solá et al. 2020). Our findings are also consistent with a short-term salinity stress experiment run at the same time (Román et al. 2023), in which no effects were observed except for a decrease in the sucrose content of apical rhizomes. Indeed, *Z. noltei* is a euryhaline species that commonly grows in estuaries where salinity fluctuations are usual (Hemminga & Duarte 2000). Thus, our results suggest that an increase in the frequency of low-salinity events will not be a critical threat for the sustainability of *Z. noltei* meadows in the near future.
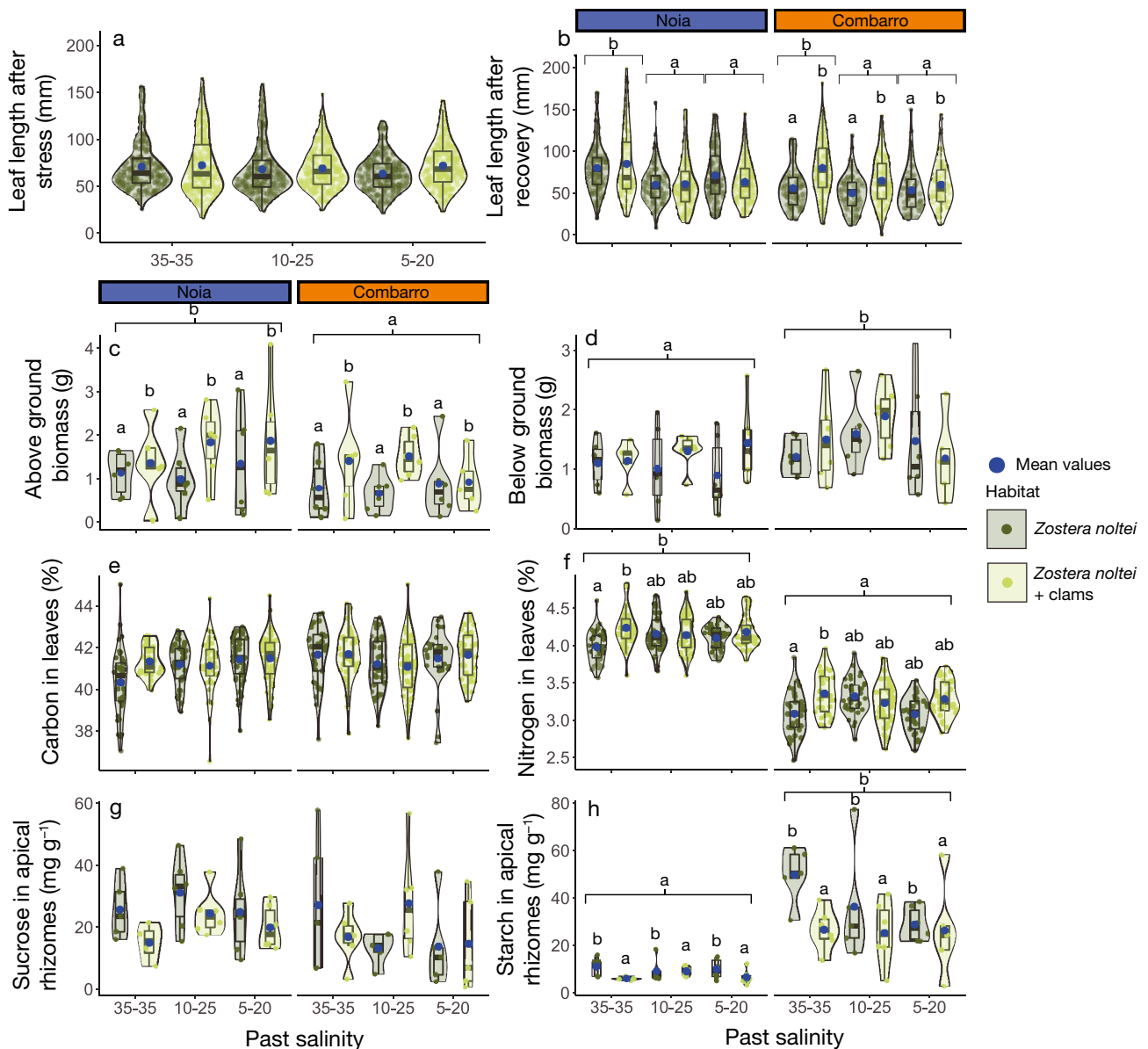
Fig. 3. Violin plots showing the responses of *Zostera noltei* in the transplant experiment in the Noia and Combarro shellfish beds. Leaf length after salinity stress in the mesocosm experiment (n = 118—120), i.e. (a) initial conditions of the transplant experiment and (b) at the end of the transplant experiment (n = 55—119). (c,d) Above- and below-ground biomass (n = 5—6), (e,f) carbon and nitrogen contents of leaves (n = 29—48) and (g,h) sucrose and starch contents of apical rhizomes (n = 6). Letters indicate differences in levels of the significant terms of the models calculated using post hoc tests

However, in *Z. noltei* transplanted with clams, the above-ground biomass increased and the starch content of apical rhizomes decreased in all past salinity treatments. This response may have been driven by clams through the release of faeces and pseudofaeces and the consequent increase in ammonium and phosphate levels (Dame 1998a, Gosling 2015), which would thus be assimilated by the seagrass and enhance the growth of above-ground biomass (Hemminga 1998, Touchette & Burkholder 2000). The

decrease in the starch content of apical rhizomes, synchronized with the increase in above-ground biomass, suggested an increasing demand for C stored in the rhizomes for the assimilation of nutrients (Jiménez-Ramos et al. 2022) and for sustaining leaf growth, particularly under light limitation (Hemminga & Duarte 2000).

The greater seagrass biomass in the presence of clams in all salinity treatments suggested facilitation effects on *Z. noltei* driven by juvenile clams. However,

Table 2. Final linear models used to test the effects of the fixed factors site (2 levels: Noia and Combarro), Past salinity (3 levels: 35—35, 10—25 and 5—20), Habitat (2 levels: *Zostera noltei* with clams and *Z. noltei*) and the random factor Basket (6 levels) on the *Z. noltei* response variables. Significant effects are highlighted in **bold**

Response variable	Factor	df	*F*	p	Random effects Variance	SD
Initial leaf length	Basket				402.845	20.071
Final leaf length[a]	Site	1, 60	9.516	**0.003**		
	Past salinity	2, 60	4.714	**0.013**		
	Habitat	1, 60	0.169	0.682		
	Habitat × Site	1, 60	3.987	**0.050**		
	Basket				269.354	16.412
Above-ground biomass	Site	1, 67	4.198	**0.044**		
	Habitat	1, 67	7.813	**0.007**		
Below-ground biomass	Site	1, 68	5.330	**0.024**		
Carbon in leaves	Basket				0.533	0.730
Nitrogen in leaves	Site	1, 62	426.131	**<0.001**		
	Past salinity	2, 62	3.429	**0.039**		
	Habitat	1, 62	9.811	**0.026**		
	Habitat × Past salinity	1, 62	3.504	**0.036**		
	Basket				0.0269	0.164
Sucrose in apical rhizomes	Site	1, 63	2.432	0.124		
Starch in apical rhizomes[a]	Site	1, 62	39.836	**<0.001**		
	Habitat	1, 62	4.940	**0.030**		
	Habitat × Site	1, 62	2.674	0.107		

[a]Models in which the variance structure was specified

Table 3. Results of final binomial generalized linear mixed model used to test the effects of the fixed factors Past salinity (3 levels: 35—35, 10—25 and 5—20), Habitat (2 levels: bare sediment and *Zostera noltei*) and the random factor Basket (6 levels) on clam survival. Significant (p ≤ 0.05) effects are highlighted in **bold**; and asterisks indicate almost statistically significant results

Species	Site	Factor	df	χ^2	p	Random effects Variance	SD
Venerupis corrugata	Noia	Past salinity	2, 28	32.384	**<0.001**		
		Habitat	1, 30	5.825	0.016		
		Habitat × Past salinity	2, 28	8.607	**0.014**		
		Basket				0.622	0.789
	Combarro	Past salinity	1, 19	3.641	0.056*		
		Basket				0.447	0.669
Ruditapes decussatus	Noia	Basket				1.290	1.136
	Combarro	Past salinity	2, 29	11.162	**0.004**		
		Habitat	1, 30	6.096	**0.014**		
		Habitat × Past salinity	2, 29	4.716	0.094*		
		Basket				1.625	1.275
Ruditapes philippinarum	Noia	Past salinity	2, 32	8.598	**0.014**		
		Habitat	1, 33	0.050	0.824		
		Habitat × Past salinity	2, 32	8.122	**0.017**		
		Basket				0.798	0.893
	Combarro	Basket				0.872	0.934

facilitation did not occur during the few days of the salinity stress treatment, indicating the need for longer observation periods to observe the positive effects of this clam—seagrass interaction. This agrees with previous findings in which the presence of *Loripes orbiculatus* Poli, 1795 caused an increase in above-ground biomass of *Cymodocea nodosa* (Ucria) Ascherson growing in polluted sediments (Cardini et

al. 2022). Indeed, long-term studies have shown similar positive interactions as in our study, for *Z. marina* with the tellinid *Macoma balthica* (Meysick et al. 2020), and for *Z. noltei* with the lucinid *L. orbiculatus* (de Fouw et al. 2022), although under non-stressful conditions and with adult clams. Our study findings revealed that even juvenile clams had a facilitative effect on *Z. noltei* in above-ground biomass in all situ-
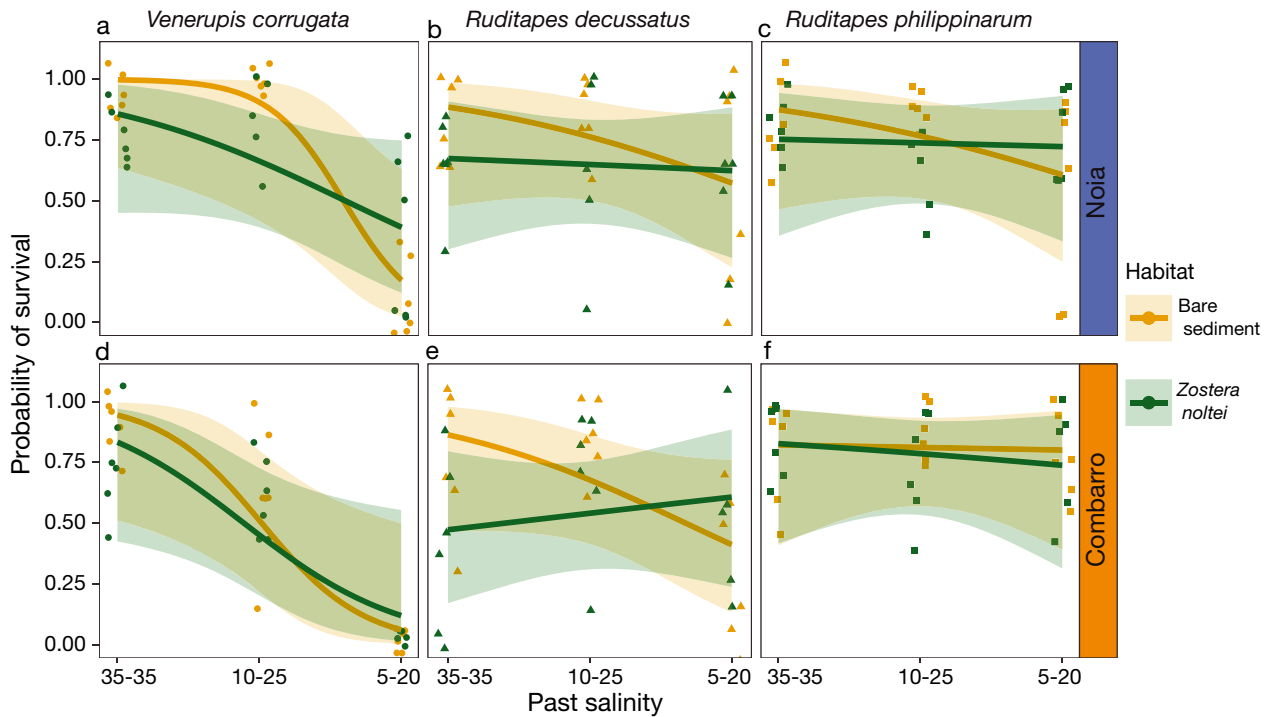
Fig. 4. (a–f) Survival of clams in bare sediment and mixed with *Zostera noltei* in the transplant experiment in the Noia and Combarro shellfish beds; points: observations; lines: data fitted by binomial generalized linear models; shaded areas: 95% confidence intervals

ations and on leaf growth when the nutrient concentration was lower in the Combarro site.

4.2. Legacy effects of low salinity on juvenile clams and their interactions with *Zostera noltei*

Overall, the previous exposure to low salinity had lethal effects on juvenile clams, observed 2 mo after the stress, especially in *Venerupis corrugata* and some individuals of *Ruditapes decussatus*, although the magnitude of responses varied between species, habitats and sites. The clams probably experienced starvation and weakening before recovery, as at the end of a short-term salinity stress experiment, the clearance rates and respiration of *V. corrugata* and *R. decussatus* were significantly lower in response to the reduced salinity (Román et al. 2023). Indeed, low-salinity stress decreases feeding rates, energy reserves, growth and immune responses, compromises reproduction and increases oxidative stress in clams (Carregosa et al. 2014a, Domínguez et al. 2020, Pourmozaffar et al. 2020, Vázquez et al. 2021, Blanco et al. 2022). Additionally, during the transplant experiment in the field, clams were exposed to high sediment temperatures typical of early summer, especially during low tide. The legacy effects of past low salinity could have enhanced the vulnerability of juvenile clams to increased temperature, even though these conditions did not occur simultaneously, as they occurred in relatively fast succession, thus probably causing synergistic effects (Gunderson et al. 2016). Low salinity and high temperature can interact, narrowing the tolerance of organisms (Dame 1998b), and they can act synergistically, for instance, increasing the mortality of *R. philippinarum* (Dethier et al. 2019) and of *R. decussatus* (Rato et al. 2022). These effects may have been greater at the Combarro site, where the 90th percentile of temperatures and cumulative heating were higher than at Noia. With the rapidly changing climate, synergistic interactions of climatic stressors could exacerbate mortality rates in juveniles, thus compromising and putting at risk the replenishment of stocks of adult-size commercial clams in shellfish beds.

Remarkably, the findings showed that *Z. noltei* plants reduced the mortality of clams previously exposed to a salinity of 5, in *V. corrugata* and *R. philippinarum* in the Noia site and in *R. decussatus* in the Combarro site, indicating positive interactions after exposure to extreme environmental conditions. This contrasted with the findings of a short-term salinity stress experiment, in which clam mortality was not reduced by the presence of *Z. noltei* (Román et al. 2023). Enhanced clam survival in the presence of sea-

grass has been generally observed in field studies (Fales et al. 2020). This has been attributed, for instance, to protection against pollution (Cardini et al. 2022) or predation (Irlandi & Peterson 1991). This effect was diminished in our study, as disturbance and predation by large predators was already inhibited by the mesh used to line the baskets, and therefore the role of seagrass to buffer temperature stress in juvenile clams (Román et al. 2022) weakened by past hyposalinity is one plausible explanation. Other potential explanations include an increase in food availability within the above-ground parts (Agawin & Duarte 2002) or enhanced oxygen supply by the rhizomes (Jensen et al. 2005). Plans that promote the maintenance of seagrass patches within shellfish beds can be beneficial for clam survival after floods or extreme rainfall events.

Clams were resilient to sublethal impacts of past salinity stress. Effects of previous exposure to low salinity on clam growth and CI were blurred during the field period, whereas the short-term salinity stress decreased shell growth at salinity levels of 5−20 (Román et al. 2023). Negative effects of low salinity on shell and flesh growth were generally observed in bare sediment, while in *Z. noltei*, no differences appeared. This may be partly due to the greater food availability on seagrass leaves and sediments below the canopy. Seagrass meadows have a greater capacity than bare sediment to retain and accumulate sestonic particles and nutrients from the water column and to transfer planktonic production to the benthos (Agawin & Duarte 2002, Barrón et al. 2006), among which microphytobenthos, phytoplankton and epiphytes are the main food sources for *Ruditapes* spp. (Machás et al. 2003, Dubois et al. 2014). Previous research has found that food availability and growth rates of filter-feeder bivalves are greater in patch edges and low-density patches than in the middle of larger and denser ones (González-Ortiz et al. 2014 and references therein). Nonetheless, this effect could not be tested in our experimental units that were transplanted to the middle of seagrass patches.

The observed survival and growth rates suggested that *V. corrugata* was the clam species most sensitive to low salinity and less resilient to past stress, while *R. philippinarum* was the most resilient. This was previously observed at biochemical and physiological levels, with greater mortality rates recorded in *V. corrugata* than in *R. philippinarum* or *R. decussatus* (Carregosa et al. 2014a,b, Domínguez et al. 2020). The siphon activity and feeding of *V. corrugata* reach inflection points at higher salinity than those of *R. philippinarum* and *R. decussatus* because the siphonal

gape in *V. corrugata* cannot isolate the animal from the surrounding water, whereas the other 2 species close their valves completely (Woodin et al. 2020 and references therein). By contrast, the greater survival and growth of *R. philippinarum* after previous stress at low salinity than that in the native species was consistent with its success in global shellfisheries (www.fao.org) and may imply an advantage under more frequent storms and floods expected to occur in the future.

4.3. Influence of environmental conditions on recovery of clams and *Zostera noltei*

The study site and, therefore, environmental conditions influenced the responses of each functional group to past stress. At the Noia site, the environmental conditions were less harsh than at Combarro, with lower immersion and emersion temperatures and greater ammonia and nitrate contents in the surface seawater, which compensated for the less saline environment. While there were no low-salinity events at Combarro, salinity fluctuated at Noia. However, the salinity fluctuations at Noia produced a cumulative exposure (SUDs) that was between the conditions of the 10 and the 35 mesocosm treatments. This cumulative exposure during the recovery period was accrued over 2 mo, whereas the exposure during the mesocosm treatments was over 6 d, so the exposure to low salinity was less intense during the recovery period than during the mesocosm experiment, which could allow organisms to acclimate to the lower salinity in the Noia site.

Accordingly, the above-ground biomass and N content of *Z. noltei* leaves were greater at Noia than at Combarro, whereas the starch content of apical rhizomes and the below-ground biomass were lower. This suggests enhancement of nitrate assimilation by the seagrass (Peralta et al. 2003) at the Noia site. Mobilization of starch in the rhizomes of *Z. noltei* supplies the energy and C necessary for the energetically costly nitrate assimilation process, particularly under darkness (Alexandre et al. 2016, Touchette & Burkholder 2000). The mobilization of starch from apical rhizomes influences the decrease in below-ground biomass, a pattern already reported in *Zostera* spp. under N fertilizing conditions (Peralta et al. 2003, Alexandre et al. 2016).

Although salinity conditions were less favourable for recovery, the lower temperature and greater food availability at Noia than at Combarro could enhance a better performance of clams. The emersion heat stress reduces the energy available for clam growth (Sobral &

Widdows 1997, Domínguez et al. 2021) and abundant nutrients in the photic zone combined with high insolation in early summer increase phytoplankton abundance (Libes 2009). In fact, the larger abundance of microphytobenthos at Noia suggests that greater nutrient inputs could enhance seston productivity. This is consistent with previous findings indicating that the density of epiphytes, phytoplankton and microphytobenthos in *Thalassia testudinum* K. D. Koenig increased under enhanced ammonium and nitrate concentrations in the water column (Cornelisen & Thomas 2006).

The clam—seagrass interactions also varied between sites, demonstrating the importance of facilitation under harsher conditions. The positive effect of clams on leaf length of transplanted *Z. noltei* was only evident at Combarro, the site where nutrient availability was lower. Given that nutrient inputs increase leaf length of its congener *Z. japonica* Ascherson & Gerbner (Han et al. 2017), the inputs of N derived from clam excretion may play a relevant role in N acquisition and growth of *Z. noltei* leaves under conditions of nutrient limitation.

5. CONCLUSIONS

The study findings show that exposure to low salinity had legacy effects on the leaf length of *Zostera noltei* but no effects on the other ecophysiological traits, whereas past low salinity negatively affected clam survival but not growth or CI. The reciprocal interactions between *Z. noltei* and clams during recovery were greater survival of clams in the presence of *Z. noltei*, and greater biomass and %N in the aboveground parts of *Z. noltei* in the presence of clams, although the magnitude and direction of interactions were site- and species-specific. *Venerupis corrugata* and *Ruditapes philippinarum* were respectively the least and the most resilient clam species.

The recovery trajectories varied with the environmental context because study sites located 100 km apart exhibited slight environmental differences, pointing out the importance of the effects of temperature and nutrient availability (Fig. 5). At the colder, more nutrient-rich site (Noia), greater biomass and %N were observed in the above-ground parts of

Fig. 5. Syntax table. Hypotheses outlined in this study, predictions derived from alternative hypotheses and results obtained

Z. noltei, the clam growth tended to be greater and facilitation of clam survival (by the presence of *Z. noltei*) after low-salinity stress appeared to be more evident than at the warmer, less nutrient-rich site (Combarro). This study thus emphasizes the problems with generalizing findings from single case studies to achieve a realistic knowledge of ecosystem dynamics under climatic stressors and to make informed predictions about changes in resilience of coastal systems.

The expected increase in heavy rainfall and flood events in the future would increase the frequency of mortality events in the juvenile clams studied long after the end of stress, whereas *Z. noltei* would not be impacted. The enhanced survival of juvenile clams below *Z. noltei* after low-salinity stress implies that seagrass provides an important ecosystem service guaranteeing the sustainability of shellfisheries, especially in estuarine areas. This could add value to (and thus favour the conservation of) the seagrass meadows where shellfish beds are located.

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