# Susceptibility of two co-existing mytilid species to simulated predation under projected climate change conditions

Jose MF Babarro[1*], María José Abad[2], Ignacio Gestoso[3], Elsa Silva[1], Celia Olabarria[4]

[1] Instituto de Investigaciones Marinas CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

[2] Grupo de Polímeros (CIT), Campus de Esteiro, C/Dr Vázquez Cabrera, s/n 15403- Ferrol (A Coruña), Spain

[3] MARE – Marine and Environmental Sciences Centre, Quinta do Lorde Marina, Sítio da Piedade, 9200-044 Caniçal, Madeira Island, Portugal

[4] Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, 36310 Vigo, Spain

*Corresponding author. Email: jbabarro@iim.csic.es

Tel.: +34 986 231930 Ext. 201; Fax: +34 986 292762

**Abstract**

Properties of the shells and byssus filaments secreted by marine mussels are affected by environmental and biotic factors. In this study, we investigated the effects of pH and temperature on shell and byssus in artificially created monospecific and mixed aggregations of the indigenous mussel *Mytilus galloprovincialis* and the invasive mussel *Xenostrobus securis.* The variability in the response of the mussels was mainly explained by species-specific interactions derived from the type of aggregation. In the mixed groups, acidic conditions caused a decrease in byssus strength in *M. galloprovincialis*, but an increase in byssus strength in *X. securis.* Increased temperature positively affected shell strength in *X. securis*, but only in mixed aggregations. Interactive effects of acidification and warming were only detected in the organic matter of shells, the strength of which decreased in *M*. *galloprovincialis* in mixed aggregations. Although the invasive mussel may be able to take advantage of changed conditions by enhancing byssal attachment, the effects that acidification has on shells may make this species more vulnerable to some predators. The study findings provide some insight into the responses of protective and attachment structures of mussels to biotic and abiotic stressors, highlighting how species-interactions may shape the future of mytilid populations.

**Keywords.** *Mytilus galloprovincialis, Xenostrobus securis*, protective structure, byssus attachment, climate change

**Introduction**

The increase in atmospheric $CO_2$ concentrations due to human activities is provoking changes in the global climate, modifying atmospheric and oceanic temperatures and sea carbon chemistry (Meehl et al., 2007), with the consequent acidification of the oceans (Doney et al., 2009). Since the industrial revolution, the concentration of atmospheric $CO_2$ has increased from 280 to 395 ppm, causing a decrease in the pH of surface ocean waters of 0.1 units and an increase in the global mean sea surface temperature of 0.7 °C (Raven et al., 2005). Such changes have modified the carbonate (dissociation) system, leading to a decrease in the concentration of carbonate ions and a consequent reduction in $CaCO_3$ saturation states of aragonite (Ω arag) and calcite (Ω calc) (Gazeau et al., 2013). The $CO_2$ concentration is expected to rise above 1100 ppm by the end of this century, possibly leading to a decrease in pH of approximately 0.3-0.4 units and an increase in the mean global sea surface temperature of between 2.6 and 4.8 °C (IPCC, 2013). These predictions for the open ocean may have even greater impacts in coastal or nearshore zones due to the possibly wider range of pH variation caused by coastal upwelling, aquaculture, urban activities and marine-terrestrial interactions, which may further exacerbate the worst scenarios predicted. Ocean acidification and warming have significant consequences at many organization levels in marine ecosystems and the combination of both may have an even stronger impact than the sum or product of the separate effects (Fabry et al., 2008; Duarte et al., 2014).

Mussels are important ecosystem engineers that dominate rocky shores worldwide and that have a significant ecological role with important implications for ecosystem functioning and coastal diversity (Borthagaray & Carranza, 2007). Moreover, some species of the family Mytilidae represent a large part of worldwide aquaculture production, especially those species belonging to the genus *Mytilus*. The calcium carbonate shell of mussels is composed of between 95 and 99.9% $CaCO_3$ arranged in two polymorphs: an outer layer of calcite (prismatic) and an inner layer of aragonite (nacreous layer), whereas the remaining mass (0.1–5 %) corresponds to the organic matrix (Marin et al., 2008). Formation of the shell (including the organic matrix) and calcification are both physiologically costly processes and may be sensitive to environmental changes, e.g. changes in pH and temperature (Duarte et al., 2014; Li et al., 2015; Gestoso et al., 2016). Although acidification has very variable effects on shell structure or growth, the findings of many studies suggest a negative impact on calcification rates in mussels (Michaelidis et al., 2005; Fernández-Reiriz et al., 2012; Gazeau et al., 2013). The effects include loss of shell integrity and functionality (Fitzer et al., 2015), which in turn may affect predator-prey interactions, making mussels more vulnerable to predators (Amaral et al., 2012). Nevertheless, no impact or even positive consequences of ocean acidification have been reported

(e.g. Ries et al., 2009), suggesting that the responses of organisms to environmental change may be species-specific (Fernández-Reiriz et al., 2012) and locally influenced (Range et al., 2014).

Much less information is available about another key material secreted by mussels, i.e. the byssal filaments that ensure a gregarious and sessile mode of life by enabling these organisms to attach to almost any surface (Waite, 2002). Recent studies of mytilids have revealed that this non-calcifying material may also be negatively affected by acidification (O'Donnell et al., 2013; Sui et al., 2015; Zhao et al., 2017). For example, the byssal threads of *Mytilus trossulus* exposed to high levels of $CO_2$ were weaker and less extensible than those of mussels exposed to control conditions (O'Donnell et al., 2013). Moreover, exposure of the mussel *M. coruscus* to low pH conditions (i.e. 7.3) negatively affected the number of byssal threads secreted, while the impact of more usual pH (i.e. 7.7) depended on $O_2$ concentration (Sui et al., 2015). Secretion of byssal threads by the internal secretory gland is also pH-dependent for a number of chemical functions involving histidine-metal crosslinks, which together with the high concentration of the modified amino acid 3,4-dihydroxyphenylalanine (Dopa) play an important role in adhesion to surfaces and self-healing after deformation (Harrington & Waite, 2007).

Temperature is another key environmental variable that affects bio-mineralization processes, growth and metabolism of mussels (Gazeau et al., 2013). Temperature often mediates physiological responses and regulates the development of life history traits via energetic costs (Barbosa et al., 2014). The allocation of available energy budget to the production and maintenance of protective structures instead of soft tissues in organisms may therefore be greatly affected by changes in temperature. As the pH and temperature of the ocean vary simultaneously, synergistic effects are highly likely (Pörtner et al., 2005; Pörtner, 2008; Kroeker et al., 2013). This has significant ecological and functional implications, although the magnitude and direction of effects may vary among species (Li et al., 2015). Together with temperature, acidification may cause a narrowing of optimal thermal windows (Pörtner & Farrell, 2008), which may increase the susceptibility of protective structures to environmental change (Gazeau et al., 2013). However, temperature does not always interact synergistically with acidification, e.g. in shell breaking force in *M. edulis* (Li et al., 2015), and it may even offset the negative effects of acidification (Kroeker et al., 2014).

Apart from the physiological and behavioural responses of mussels, species interactions may modulate the effects of environmental conditions at individual and assemblage levels, highlighting the complexity of mussel responses when various environmental factors act together (Li et al., 2015; Gestoso et al., 2016). For example, under acidic conditions *M. galloprovincialis* survived better in mixed aggregations with *X. securis* than in monospecific aggregations (Gestoso et al., 2016). The former species also showed a greater resistance to heatwave stress when co-occurring with *X. securis* in mixed aggregations (Olabarria et al., 2016). Temperature can also alter predator-prey interactions by modifying prey abundance and thus altering the activity of predators (Dahlhoff et al., 2001; Queirós et al., 2015). Overall, species interactions may make the responses of species less predictable and even closely-related species may show opposite responses in multispecies

assemblages due to alterations in the effectiveness of predation in the most tolerant species (Hale et al., 2011; Kroeker et al., 2011).

In the present study, we carried out a mesocosm experiment to explore the possible interactive effects of pH and temperature on two distinct protective structures, i.e. calcified and non-calcifying byssal filaments, in two co-existing mussel species, the invasive black pygmy *Xenostrobus securis* (Lamarck, 1819) and the native, commercially-important *M. galloprovincialis* (Lamarck, 1819). The species co-occur on rocky shores of the inner areas of Galician Rias Baixas (NW of Spain), but the abundance of each varies greatly along the estuarine gradient, mainly in relation to the salinity (Gestoso et al., 2012). Although both species belong to the same family, *Mytilidae*, they differ in their biological and ecological traits (Babarro & Lassudrie, 2011, Babarro & Abad, 2013) and have been reported to respond differently to changes in pH and temperature (Gestoso et al., 2015; 2016). We also investigated whether the mussel responses were both shaped by the presence of conspecific or heterospecific mussels. Most research has focused on the effects of climate change on mussels in isolation (but see Gestoso et al. 2016), and less information is available for predicting the impact of species interactions on the responses of individuals. Finally, after exposing the mussels to various conditions of pH and temperature, we tested the resistance of the shells to simulated mechanical and chemical stress of one of the most common benthic predators of mussels, i.e. the dog whelk *Nucella lapillus*. Dogwhelks, like other muricids, drill holes in shells of their prey, usually either above the most nutritious part or the most easily accessible point of the shell (Morton, 2008). They attack different calcified shelled species with a very efficient predatory strategy that involves drilling the prey shell by both mechanical and chemical processes before ingesting the internal soft tissues (Gabriel, 1981). Potential changes in shell properties in terms of composition and functionality derived from changes in pH or temperature would make mussels more vulnerable to attack by predators. This is especially important for comparison of these two mytilids, the shells of which differ considerably in terms of inorganic carbon content (Gestoso et al., 2016).

**Materials and Methods**

*Artificially assembled aggregations*

Individual mussels of similar size (*M. galloprovincialis*: 33.13 ± 0.24 mm shell length; *X. securis*: 33.50 ± 0.20 mm shell length; mean ± SE) were collected from the mid-intertidal area of a rocky shore in the inner part of the Ria de Vigo, NW Spain (42º 18’ 43” N, 8º 38’ 9” W). The mussels were transported to the laboratory where epibionts were removed from the shells and byssal threads were carefully cut from the ventral margin. The mussels were then allowed to establish primary attachment to experimental units, i.e. PVC plates (15 x 15 x 0.5 cm) covered with biodegradable cotton mesh. Three types of aggregations of similar densities to those observed in the field were established: monospecific aggregations of *M. galloprovincialis* (14 individuals),

monospecific aggregations of *X. securis* (10 individuals) and mixed aggregations (7 individuals of *M. galloprovincialis* and 5 individuals of *X. securis*).

*Mesocosm system*

The experiment was carried out in the CIIMAR Coastal Biodiversity Laboratory in Porto (Portugal). Eight tanks (350-L), each equipped with its own mechanical and biological filter system, were filled with 1 µm filtered-seawater. Seawater temperature and pH were orthogonally manipulated to produce the desired climate change conditions (IPCC, 2013) with two different levels of each: pH 8.00 and 7.65 and temperature 16 °C and 21 °C. Each treatment level was replicated in two randomly-positioned tanks, and seven replicates of each type of mussel aggregation (3 types) were randomly placed in each tank. The seawater in the tanks was renewed once a week. The pH was manipulated by diffusing pure $CO_2$ from a gas tank to the experimental tanks through a pH stat system (Aqua Medic AT Control) by opening or closing a solenoid valve when the pH readings in the experimental tanks deviated by 0.1 unit from the predetermined set points (see Gestoso et al., 2016 for detailed experimental set-up). The temperature in each tank was controlled with titanium aquarium chillers, which were also fitted with UV sterilizers (TECO TC-15). Mean pH, temperature, salinity, dissolved inorganic carbon (DIC), $CO_2$ pressure and total alkalinity for each treatment level are shown in Table 1, along with the saturation state for both calcite ($\Omega_{calc}$) and aragonite ($\Omega_{arag}$). Temperature and pH were continuously monitored using dedicated electrodes and the data-logger function of the controller. The pH electrodes were standardized against Tris seawater buffer (ionic strength, 0.7 M). Salinity was checked at least three times a week with a refractometer, and water from each tank was sampled twice a week for analysis of total dissolved inorganic carbon (TDIC). Samples were analysed with a Licor LI-7000 CO2 analyzer within 3 days of collection, and TDIC was then partitioned into CO2, bicarbonate, alkalinity, and carbonate from salinity, temperature and pH by using the Matlab program csys.m (Zeebe and Wolf-Gladrow, 2001). Calcium concentration was estimated from the salinity according to the concentrations of ions in seawater (Pilson, 2013). The saturation states of calcite (Ωcalc) and aragonite (Ωara) were calculated using the thermodynamic solubility product of calcite and aragonite following Morse et al. (1980).

Mussels were held under ambient pH conditions (pH 8.00) at 16 °C for 7 days prior to the experiment. The experiments lasted for 22 days, during which mussels were fed (3 % of total tissue dry-weight) once a day, every two days. Feeding was stopped 24 h before the measurements started.

*Shell characteristics: thickness, compressive strength and composition*

Shell thickness and compressive force were measured in the left valves of 5 to 8 mussels for each type of aggregation and treatment level. Shell thickness was measured with calipers (Mitutoyo 0−25 ± 0.01 mm) at the highest point of each valve, i.e. where the force was applied (see below). The compressive force required to break the shell was measured with a universal testing machine (Instron 5566) by applying a 1 kN cell load at an

extension rate of 2 mm $s^{-1}$ (see Babarro & Abad, 2013). Each individual mussel was placed horizontally with the shell edge on a flat surface, and a compressive force was then applied with a 2 mm diameter steel tip placed on the curved surface at the highest point used to measure shell thickness. Load displacement curves up to shell breaking point were constructed, and shell strength was calculated from the maximum force identified in the curves. Although this does not provide absolute values of the compression strength of shells, the values obtained can be used to compare the mechanical functionality of shells exposed to different combinations of factors at different levels.

In addition, 6 mussels were sampled from each type of aggregation and treatment level for analysis of the organic (periostracum) content of shells (Addadi et al., 2006). Organic content was determined using the gravimetric method, by calculating the organic matter loss after calcination at 600 °C. The inorganic carbonates were measured by inductively coupled plasma optical emission spectrometry (ICP-OES).

*Shell resistance to mechanical and chemical stress*

Shells of 16 individuals of each species were randomly selected from the four environmental tests and analysed to determine their resistance to different chemical and mechanical stresses simulating attacks by dogwhelks. Only shells of mussels from mixed aggregations were considered because the most significant changes in shell composition were observed in this type of aggregation. Empty shells were dried and weighed to the nearest 0.001 g before and after being exposed to the different stress factors. Specifically, the effects of 0.06M EDTA, 0.05 mM hydrochloric acid (pH 4.0) and a non-specific protease (papain from papaya latex Sigma P3375, 1 g/100mL, at pH 7.16 with 1N NaOH) were tested during a period of 24 h, with empty shells placed in 50 ml glass flasks (see Gabriel, 1981 for detailed description of procedure). In addition, abrasion was simulated by placing empty shells in 3.5 L glass flasks each containing 10.5 g of fine grade carborundum 80 powder (165 µm; Fisher Chemical) for 7 days. Water motion was simulated by magnetic stirrers placed at the bottom of the flasks, and empty shells were hung and maintained in the water column. All tests were carried out at room temperature, except the protease assay, which was conducted at 37 °C in an isothermal bath.

*Attachment strength*

Between 10 and 20 mussels were randomly selected from each type of aggregation and environmental condition to measure the attachment strength of individuals. Only non-manipulated individuals were used to measure byssus strength, because the byssal filaments are interconnected among the individuals in aggregations and the filaments of mussels adjacent to those dislodged during the measurements may be weakened.

Mussel detachment force was measured in immersion conditions to prevent modification of the mechanical properties of byssal filaments exposed to the drying effects of air. Attachment force was measured by connecting the shell to a spring scale (Digital Force Gauge DN431 with peak hold indication, resolution of

0.01 N) fitted to custom-made forceps. The spring scale was pulled perpendicularly (normal) to the substratum until the mussel was dislodged (Babarro & Comeau, 2014). Care was taken to avoid disturbing neighbouring mussels when individual mussels were dislodged. In all cases, selected mussels were attached primarily to the PVC plates.

*Statistical analysis*

Changes in shell thickness, compressive force, organic matter content of shells and byssus detachment strength were analysed separately for each species by analysis of variance (ANOVA) models including three orthogonal fixed factors: pH (two levels: 7.65 and 8.0), Temperature (Temp, two levels: 16 °C and 21 °C) and Aggregation (Agg, two levels: monospecific and mixed). Tank was a random factor nested in the pH x Temperature interaction. However, because the tank factor was not significant ($p>0.25$, data not shown), tanks were pooled in all subsequent analyses. After exposure of mussels to different environmental conditions, changes in shell weight due to chemical and mechanical stress were analysed separately for each species by an ANOVA including two orthogonal fixed factors: pH and temperature.

Normality and homogeneity of variances were tested by Shapiro-Wilk´s W and Levene tests, respectively. Whenever the assumptions of analysis of variance were violated, data were log or rank-transformed (Conover, 2012). Homogeneous groups were established *a posteriori* with the Bonferroni adjusted level for distinct sample sizes in multiple comparisons. All values shown in the figures are means ±SD. All analyses were performed with STATISTICA 7.0 software (Tulsa, OK 74104 USA).

**Results**

*Shell characteristics: thickness, compressive force and organic content*

Neither pH nor temperature had significant effects on shell thickness, which only differed between the two mussel species (Table 2). Shells of *M. galloprovincialis* were thicker (0.83 ±0.05 mm, [0.77, 0.90 range in mm]; $p<0.001$) than those of *X. securis* (0.52 ±0.03 mm, [0.46, 0.57]), irrespective of the aggregation considered.

In general, shells of *M. galloprovincialis* were stronger (146.81 ±14.86 N, [130, 170 range in N]; $p<0.001$) than those of *X. securis* (85.28 ±11.14 N, [71, 104]) due to differences in thickness (Figures 1A-D). The compressive strength of shells varied between aggregations especially in the case of the invasive species (Table 2; Figures 1C-D). The compressive strength of *X. securis* shells increased when mussels were reared at higher temperature, but only in mixed aggregations. By contrast, although marginally, compressive strength of *M. galloprovincialis* shells tended to increase in mixed aggregations at lower temperature (i.e. Temp x Agg; Table 2; Figure 1C-D).

The organic content of shells varied between the two mussel species, with higher values for *X. securis* (6.64 ±0.65 %) than for *M. galloprovincialis* (3.72 ±1.05; Figure 2A-D). The organic content of shells of *M. galloprovincialis* reared at higher temperature increased, but only in the individuals in mixed aggregations (Temp x Agg; $p<0.05$; Table 3; Figure 2A-B). Moreover, the organic content of *M. galloprovincialis* shells decreased when the mussels were reared under acidic conditions at high temperature (pH x Temp; $p<0.05$; Table 3; Figure 2A-B). In the case of *X. securis*, an increase was observed in the organic content of shells of mussels exposed to ambient (control) pH conditions, although only in mixed aggregations (pH x Agg; $p<0.05$; Table 3; Figure 2C-D).

*Shell resistance*

Exposure to EDTA (0.06 M) caused greater loss of shell weight (range 14-20%) than the other stress factors such as abrasion (3-5%) and exposure to HCl and protease (< 1%) (Figures 3 A-H). Specifically, EDTA caused the greatest weight loss in shells of *M. galloprovincialis* previously exposed to conditions of low pH and temperature (pH x Temp; $p<0.05$; Table 4; Figure 3A). No effect of the environmental parameters was observed in *X. securis* shells treated with EDTA (0.06 M). Weight loss in *X. securis* shells treated with HCl (pH 4) was noted for both the low pH and low temperature treatment levels, independently (Table 4; Figure 3D). By contrast, these environmental parameters did not affect *M. galloprovincialis* shells (Table 4; Figure 3C). Exposure to protease caused greater weight loss in shells of the invasive species exposed to higher temperature (Table 4; Figure 3F), but no effect of environmental parameters was observed for *M. galloprovincialis* shells (Figure 3E). Abrasion had a significant impact on shells of the invasive species, which lost more weight when the mussels were previously exposed to low temperature (Table 4; Figure 3H). Abrasion caused greater loss of weight of *M. galloprovincialis* shells previously exposed to low pH (Table 4; Figure 3G).

*Byssus attachment strength*

Environmental conditions affected byssus attachment strength, although this varied depending on the mussel species considered and type of aggregation (Table 5; Figure 4A-D). The attachment strength of *M. galloprovincialis* decreased when mussels were reared at low pH and temperature (i.e. significant interaction pH x Temp; $p<0.01$; Table 5; Figure 4A-B). The attachment strength of the invasive *X. securis* increased significantly when mussels were exposed to low pH, irrespective of temperature, but only in the mixed aggregations (pH x Agg; $p\sim0.01$; Table 5; Figure 4C-D).

**Discussion**

The study findings demonstrated that pH and temperature significantly affect the response variables considered, with the exception of shell thickness. They also showed interactive effects of both factors in several cases, as reported in other studies on shelled bivalves (Kroeker et al., 2013; Li et al., 2015). The responses also varied significantly in relation to species and aggregation, highlighting the importance of species interactions in shaping responses to environmental changes (Fabricius et al., 2011; Hale et al., 2011; Gestoso et al. 2015; 2016). In general, the invasive species *X. securis* performed better under acidic conditions than the indigenous mussel, as indicated by the responses of several parameters (shell strength, organic content of shell and byssus strength). Although shell thickness was not affected by the environmental conditions, the variation in compressive strength and organic content of shells indicate that *X. securis* is more resistant than *M. galloprovincialis* to environmental change. Although marginal, changes in shell compressive force occurred at high temperature, with opposing trends in *M. galloprovincialis* and *X. securis*. On the other hand, a significant decrease in the organic content of the *M. galloprovincialis* shells occurred under conditions of low pH and high temperature, but not for the invasive *X. securis*.

Intertidal organisms adapt to wide variations in environmental factors on a daily basis, e.g. frequent emersion processes and internal acidosis, which they cope with by having a compensatory mechanism to maintain homeostasis (Chaparro et al., 2009). This can make individuals more resistant to environmental stressors such as acidification and increased temperature (Scanes et al., 2017). The study findings indicate that the invasive *X. securis* is more resistant than *M. galloprovincialis*. We expected that *X. securis*, a gaping species, would be energetically more efficient (i.e. with less oxygen debt after re-immersion) and better prepared to cope with additional stressors. Indeed, previous studies on these species have indicated better physiological performance of the invasive species under environmentally stressful conditions (Gestoso et al., 2016; Olabarria et al., 2016). The environmental conditions of the original habitats where mussels were collected e.g. past history of both species may be important in shaping the responses of protective structures to environmental conditions tested in this study. For example, elevated temperature (up to 25°C) enhanced the negative effect of acidification on biomineralization and metabolism of *M. edulis* (Li et al., 2015), although the *M. galloprovincialis* shells were thicker under conditions of moderate warming (20°C), irrespective of $CO_2$ levels (7.69-8.06) (Koeker et al., 2014). The mechanical properties of aragonite-type and calcite-type shells, of respectively *Mercenaria mercenaria* and *Crassostrea virginica*, showed no impact of moderate hypercapnia (~800 µatm $pCO_2$) in mussels acclimated at 22 °C (Ivanina et al., 2013). However, other surveys did not show any negative effects of acidification on biomineralization processes neither on shell functioning, i.e. the capacity to maintain crystallographic orientation and structural integrity, despite species-specific responses of bivalves (Gazeau et al., 2007; Ries et al., 2009) and the fact that some organisms are still able to secrete shell under very low pH conditions (Hiebenthal et al., 2013; Gazeau et al., 2013). In *M. edulis*, the shells may dissolve at pH 7.5 or lower (Gazeau et al., 2007; Michaelidis et al., 2005), i.e. under more intense acidification

than tested here. Mussels seem to tolerate pH fluctuations and even a decrease in the aragonite saturation state within a wider range of temperatures that they naturally experience in the field (Kroeker et al., 2014). In the present study, the mesocosm system was not undersaturated with respect to calcite or aragonite, which may explain why the composition of the shells remained unaltered (see also Range et al., 2012; Gestoso et al., 2016). The positive (although marginal) effect of temperature on shell strength in the invasive species may be partly due to the upper thermal tolerance of mussels, i.e. above 25°C (Kimura & Sekiguchi, 2009), which is still very different from that tested in our experiment. Exposure of *X. securis* to elevated temperature improved shell strength rather than exacerbating any negative effects of acidification, which is consistent with previous findings in *M. edulis* (Li et al., 2015). The fact that differences in shell strength were not accompanied by changes in shell thickness suggests that other factors may play a role in the response of this protective structure. For example, the crystallographic orientation of the mineral units that form part of the shell corresponds to changes in temperature; in addition, other micro-structural changes such as size and elongation of prismatic structural units have been positively correlated with seawater temperature (Milano et al., 2017).

The high organic content of *X. securis* shells is probably a key factor influencing the greater capacity of this species to withstand environmental stress because the organic content plays a major role in protecting shells from acidification (see Ries et al., 2009). Interestingly, the organic content of the *M. galloprovincialis* shells decreased under acidic and warmer conditions. By contrast, the *X. securis* shells were not affected by these conditions and even showed an increase in organic content when present in mixed aggregations at ambient pH. However, *X. securis* shells with a higher organic content also underwent greater weight loss after the simulated predatory attacks. Ocean acidification may have a greater impact on shell dissolution than on shell deposition (Nienhuis et al., 2010) and, consequently, lower pH may explain significant changes in shell weight. Low pH (7.8) has been reported to significantly affect the organic matrix and biomineralization rates in *M. edulis* (Li et al., 2015). Indeed, acidification exacerbated the weight loss of shells subjected to simulated predatory attacks, with *X. securis* suffering the greatest weight loss, especially at low pH (hydrochloric acid) but also after simulated predatation (protease) at higher temperature. Aragonite-type shells are thought to be more susceptible to dissolution, mainly at low temperature (Mackenzie et al., 2014).

Environmental conditions also affected byssus attachment strength, although the responses of the two species varied greatly. Acidification reduces byssus strength in *M. galloprovincialis* within the predicted range of climate change projections and the effect was not exacerbated by high temperature. This indicated that byssal attachment was not more susceptible to seawater acidification at higher temperature. Similar responses were obtained when shell breaking force of *M. edulis* was analysed after exposure of mussels to acidic and warming conditions (Li et al., 2015), and therefore warming may offset the negative effect of acidification on byssus strength (see also Kroeker et al., 2014). Negative impacts of acidic conditions on mussel attachment strength due to an increase in the decay of number of threads and a decrease in mechanical

performance have also been reported for different mytilids (O´Donnell et al., 2013; Sui et al., 2015; Zhao et al., 2017). *M. galloprovincialis* reared under low pH and high temperature, but still within optimal thermal window, will probably display an increase in the physiological activity, including byssogenesis rate, with a positive impact on attachment strength. Similar buffer or compensatory mechanisms of elevated temperature within predicted increasing $pCO_2$ conditions have been reported for material properties of mussel shells (Fitzer et al., 2017). Mussels may compensate for the weakened byssal threads secreted at low pH by up-regulating expression of some byssal proteins (Zhao et al., 2017), and increased temperature may have played a role in this aspect. The invasive mussel showed the opposite trend, with an increase in byssus attachment strength under acidification, although the effect was only significant in mixed aggregations, thus highlighting the need to consider multiple-species interactions in more detailed analyses. A lack of any effect of acidification on byssus secretion has also been reported for the oyster *Pinctada fucata* (Welladsen et al., 2010). The lack of effect of low pH on mussel attachment strength may be partly due to the large number of byssal threads secreted by *X. securis*, i.e. up to 1000 filaments (Babarro & Lassudrie 2011) compared to the 20-50 filaments secreted by *M. galloprovincialis*. Even if low pH had a negative impact on the performance and/or number of byssal threads, the invasive species still secretes a very large number of threads that could compensate for potential negative impacts of acidification.

Species interactions played a role in the responses of mussels to environmental conditions in relation to the byssus and shell strength and the organic matter content of shells. Previous studies have indicated that the negative effects of environmental conditions can be ameliorated by interactions between species (Hale et al., 2011; Jurgens & Gaylord, 2016; Olabarria et al., 2016), which suggest that responses of species are not simply additive and the dynamics of the combinations are not linear (Walther, 2010). Here, we found that mussels in mixed aggregations performed better in relation to shell strength (specifically for *X. securis* at high temperature) and byssus attachment strength (for *X. securis* at low pH). However, the opposite was also noted for the organic content of shells in mussels from mixed assemblages. Therefore, our findings showed that interspecific interactions may have an important effect on the magnitude of the impact of environmental stressors on mussel responses and complement the findings of Gestoso et al. (2016) in other mussel responses, such as survivorship, growth, condition index and shell composition. Indeed species interactions often determine responses to environmental stressors and patterns of recovery from disturbances (Montoya & Raffaelli, 2010). Interactions between new species assemblages resulting from invasion by exotic species are of special interest, as novel species interactions may become increasingly decoupled (Hughes, 2012) and changes in these relationships may alter community structure and composition (Angert et al., 2013).

In conclusion, acidification and warming had interactive effects on the mussel-related parameters considered, with a greater impact on the byssus attachment strength. This reveals that other biological structures rather than shell can be more vulnerable to changes in pH and temperature at least under short-term, acute stress. Results also indicated that both species responded differently to environmental stressors,

with *X. securis* appearing to be the most resistant. *M. galloprovincialis* may be at a disadvantage relative to the invasive *X. securis* because weakened byssal attachment may represent a challenge for this species, especially in suspended-culture mussel farms. However, despite the better performance of the shell and byssus structures of *X. securis*, this species may be more vulnerable to attack by predatory drilling species. Overall, the most significant losses in shell weight were observed for the invasive *X. securis* previously exposed to low pH and warming seawater conditions and then treated with acid (HCl) and protease stressors, respectively. The impact of abiotic and biotic stressors and their potential interactions within multiple-species assemblages must be addressed in future studies in order to improve our understanding of the impact of climate change on marine ecosystems (see Queirós et al. 2015). The findings of the present study confirm that the impact of climate change is complex and influenced by species-specific interactions, making it necessary to consider different response variables in order to analyse future effects on the structure and functioning of mussel communities.

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**Table 1**. Mean (±SD) values of carbonate chemistry variables in seawater for each treatment during the mesocosm experiment.

| Treatment | combination | pH n=42 | Temperature (°C) n=42 | Salinity (‰) n=18 | DIC (µmol C $kg^{-1}$) n=12 | pCO2 (ppm) n=12 | TA (µmol $kg^{-1}$) n=12 | ΩCa n=12 | ΩAra n=12 |
|---|---|---|---|---|---|---|---|---|---|
| pH 8 | 16 °C | 7.98 ± 0.01 | 16.04 ± 0.01 | 36.42 ± 0.08 | 1850.54 ± 6.19 | 566.12 ± 4.64 | 1979.75 ± 15.78 | 3.00 ± 0.03 | 1.88 ± 0.02 |
| pH 8 | 21 °C | 8.01 ± 0.01 | 20.67 ± 0.02 | 36.42 ± 0.06 | 1769.74 ± 6.79 | 543.21 ± 4.49 | 2100.35 ± 19.82 | 4.07 ± 0.05 | 2.54 ± 0.03 |
| pH 7.65 | 16 °C | 7.62 ± 0.05 | 16.46 ± 0.03 | 36.14 ± 0.02 | 2009.27 ± 100.80 | 1371.33 ± 15.20 | 2160.18 ± 24.82 | 1.73 ± 0.02 | 1.09 ± 0.01 |
| pH 7.65 | 21 °C | 7.63 ± 0.07 | 21.36 ± 0.01 | 36.46 ± 0.04 | 1876.00 ± 75.86 | 1319.81 ± 23.17 | 2077.76 ± 19.63 | 2.04 ± 0.03 | 1.27 ± 0.02 |

**Table 2.** Three-way ANOVAs to determine the effect of pH, temperature (Temp) and aggregation (Agg) on shell thickness and compressive force values of the mussel shells. See main text (Materials and Methods) for specific details of each factor. Shell responses were rank-transformed prior to the analyses (see main text). Marginal and significant differences are indicated with the symbol $^{+}$ and in bold, respectively.

| ***M. galloprovincialis*** | shell thickness | | | shell compressive force | |
|---|---|---|---|---|---|
| Source of variation | df | F | p | F | p |
| pH | 1 | 0.023 | 0.879 | 0.980 | 0.329 |
| Temp | 1 | 0.303 | 0.586 | 0.015 | 0.904 |
| Agg | 1 | 0.254 | 0.617 | 1.269 | 0.267 |
| pH x Temp | 1 | 0.639 | 0.429 | 3.154 | 0.084 |
| pH x Agg | 1 | 0.003 | 0.953 | 0.034 | 0.854 |
| Temp x Agg | 1 | 0.642 | 0.428 | 4.267 | **0.046**$^{+}$ |
| pH x Temp x Agg | 1 | 2.772 | 0.105 | 0.036 | 0.851 |
| Error | 36 | | | | |

| ***X. securis*** | shell thickness | | | shell compressive force | |
|---|---|---|---|---|---|
| Source of variation | df | F | p | F | p |
| pH | 1 | 0.017 | 0.897 | 0.001 | 0.976 |
| Temp | 1 | 0.786 | 0.381 | 0.403 | 0.529 |
| Agg | 1 | 0.006 | 0.939 | 7.252 | **0.010** |
| pH x Temp | 1 | 0.876 | 0.355 | 1.387 | 0.247 |
| pH x Agg | 1 | 0.121 | 0.730 | 0.122 | 0.729 |
| Temp x Agg | 1 | 0.275 | 0.603 | 5.335 | **0.027** |
| pH x Temp x Agg | 1 | 0.4 | 0.531 | 0.158 | 0.693 |
| Error | 36 | | | | |

**Table 3.** Three-way ANOVAs to determine the effect of pH, temperature (Temp) and aggregation (Agg) on organic matter of the mussel shell. Dependent variable was log-transformed prior to the analyses. Significant differences are indicated in bold.

| | *M. galloprovincialis* | | | *X. securis* | |
|---|---|---|---|---|---|
| Source of variation | df | F | p | F | p |
| pH | 1 | 4.575 | **0.038** | 0.007 | 0.934 |
| Temp | 1 | 0.194 | 0.662 | 0.208 | 0.651 |
| Agg | 1 | 0.032 | 0.859 | 3.935 | 0.054 |
| pH x Temp | 1 | 5.744 | **0.021** | 0.032 | 0.859 |
| pH x Agg | 1 | 3.136 | 0.084 | 5.131 | **0.028** |
| Temp x Agg | 1 | 5.988 | **0.019** | 1.639 | 0.208 |
| pH x Temp x Agg | 1 | 2.538 | 0.119 | 0.258 | 0.614 |
| Error | 40 | | | | |

**Table 4**. Two-way ANOVAs to determine the effect of pH and temperature (Temp) on weight loss values (%) in shells of mussels from mixed aggregations after exposure to distinct chemical and mechanical actions. See main text (Materials and Methods) for specific details. All response variables were log-transformed prior to the analyses. Significant differences are indicated in bold.

| ***M. galloprovincialis*** | | EDTA | | HCl | | protease | | abrasion | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | df | F | p | F | p | F | p | F | p |
| pH | 1 | 0.006 | 0.942 | 0.068 | 0.798 | 0.179 | 0.680 | 7.213 | **0.020** |
| Temp | 1 | 0.091 | 0.768 | 3.355 | 0.092 | 1.620 | 0.227 | 0.214 | 0.652 |
| pH x Temp | 1 | 6.377 | **0.027** | 0.926 | 0.355 | 1.098 | 0.315 | 1.345 | 0.269 |
| Error | 12 | | | | | | | | |

| ***X. securis*** | | EDTA | | HCl | | protease | | abrasion | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | df | F | p | F | p | F | p | F | p |
| pH | 1 | 1.346 | 0.268 | 7.721 | **0.017** | 0.001 | 0.970 | 3.739 | 0.077 |
| Temp | 1 | 1.370 | 0.265 | 7.879 | **0.016** | 7.370 | **0.019** | 10.371 | **0.007** |
| pH x Temp | 1 | 2.971 | 0.110 | 3.358 | 0.092 | 1.083 | 0.318 | 2.803 | 0.120 |
| Error | 12 | | | | | | | | |

**Table 5.** Three-way ANOVAs to determine the effect of pH, temperature (Temp) and aggregation (Agg) on byssus attachment force of mussels. See main text (Materials and Methods) for specific details of each factor. Data were rank-transformed prior to the analyses (see main text). Marginal and significant differences are indicated with the symbol $^{+}$ and in bold, respectively.

| | *M. galloprovincialis* | | | *X. securis* | | |
|---|---|---|---|---|---|---|
| Source of variation | df | F | p | df | F | p |
| pH | 1 | 4.290 | **0.041**$^{+}$ | 1 | 1.022 | 0.315 |
| Temp | 1 | 0.211 | 0.647 | 1 | 2.401 | 0.124 |
| Agg | 1 | 0.007 | 0.935 | 1 | 2.869 | 0.093 |
| pH x Temp | 1 | 6.919 | **0.009** | 1 | 0.063 | 0.803 |
| pH x Agg | 1 | 0.018 | 0.892 | 1 | 6.685 | **0.010** |
| Temp x Agg | 1 | 0.894 | 0.347 | 1 | 1.600 | 0.209 |
| pH x Temp x Agg | 1 | 2.709 | 0.103 | 1 | 0.001 | 0.973 |
| Error | 99 | | | 97 | | |

**Figure legends.**

**Figure 1**. Shell compressive strength of *M. galloprovincialis* (A and B) and *X. securis* (C and D) shells after being exposed to different pH and temperature conditions. Left (A and C) and right (B and D) letters correspond to monospecific and mixed aggregations, respectively. Results of low and high temperatures are shown with black and white columns (*M. galloprovincialis*), and horizontal and vertical degraded columns (*X. securis*), respectively. Values correspond to means (± 1 SD).

**Figure 2**. Organic content of the shells of *M. galloprovincialis* (A and B) and *X. securis* (C and D) exposed to different pH and temperature conditions. See legend of Figure 1 for different letters (aggregations) and column colours and styles (temperature). Values correspond to means (± 1 SD).

**Figure 3**. Shell weight loss (%) in mussels exposed previously to different pH and temperature conditions, and treated with several chemical and mechanical stressors simulating attacks by dogwhelks (A-B: EDTA 0.06M; C-D: HCl pH=4; E-F: Protease; G-H: Abrasion). All shells considered were from mussels in mixed aggregations. Left and right letters correspond to *M. galloprovincialis* and *X. securis*, respectively. See legend of Figure 1 for different column colours and styles (temperature). Values correspond to means (± 1 SD).

**Figure 4**. Byssus attachment strength of *M. galloprovincialis* (A and B) and *X. securis* (C and D) exposed to different pH and temperature conditions. See legend of Figure 1 for different letters (aggregations) and column colours and styles (temperature). Values correspond to means (± 1 SD).

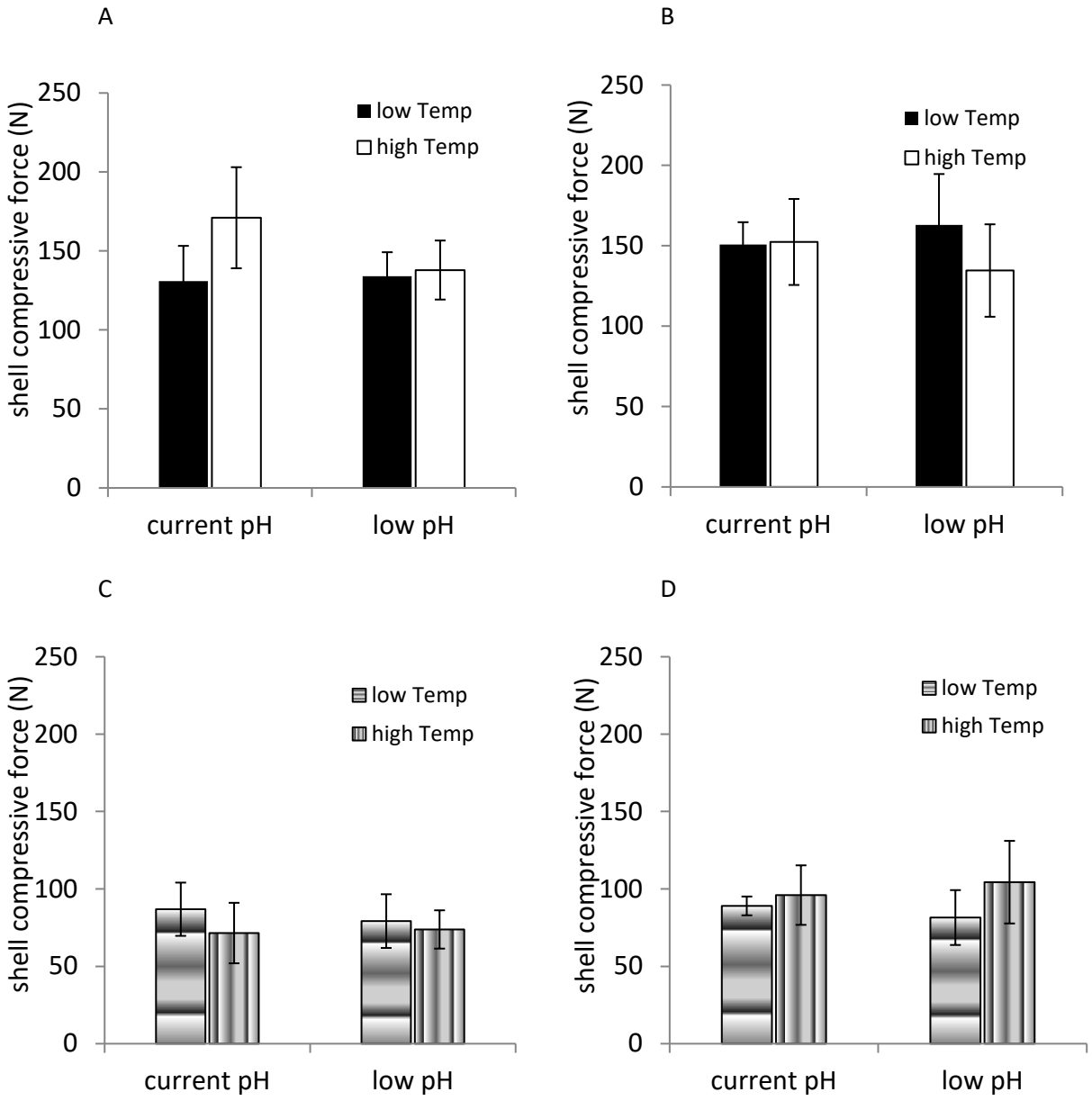
A
B
C
D
shell compressive force (N)
250
200
150
100
50
0
low Temp
high Temp
current pH
low pH

Figure 1

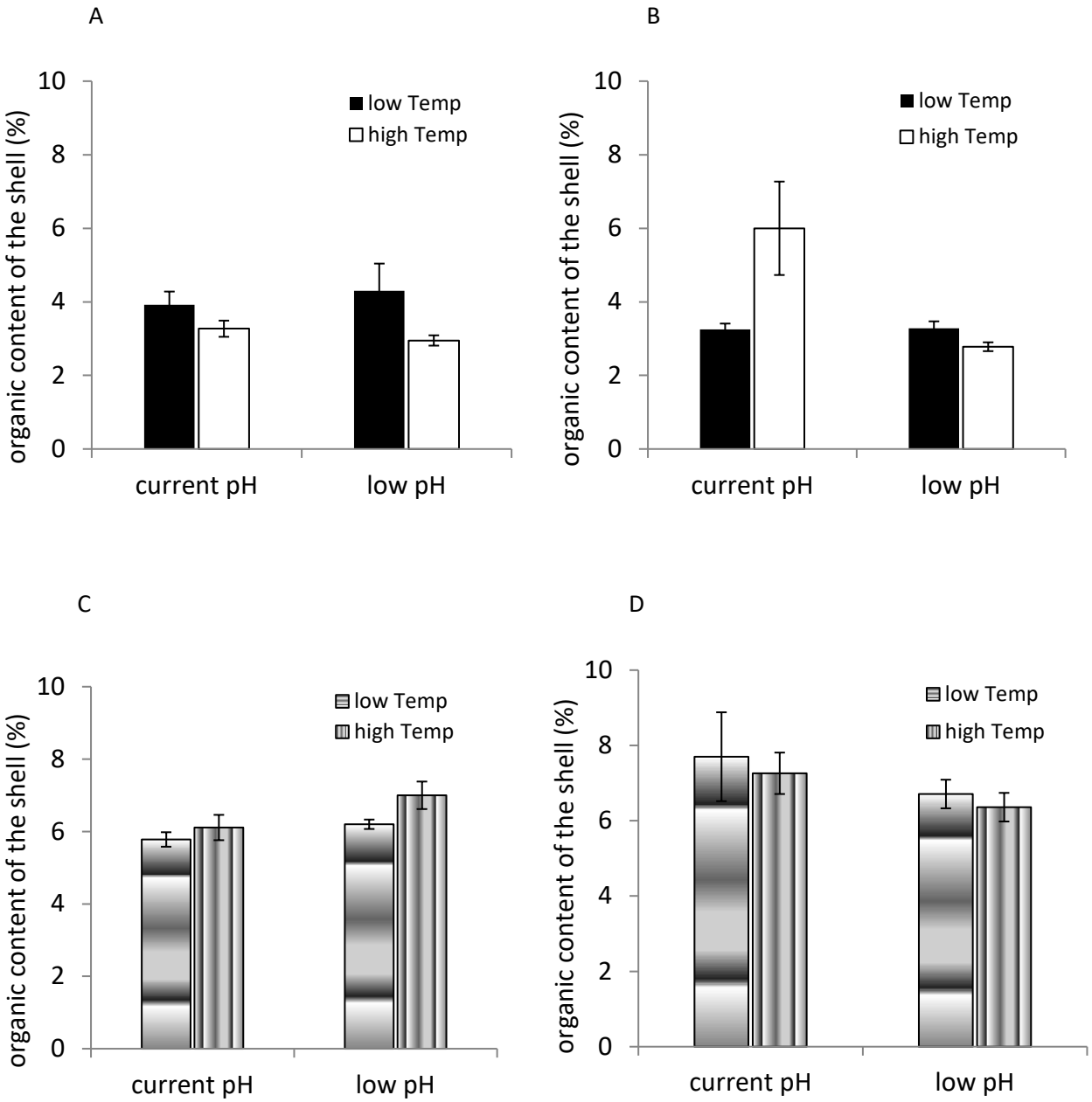
A
B
C
D
organic content of the shell (%)
low Temp
high Temp
current pH
low pH
0
2
4
6
8
10

Figure 2

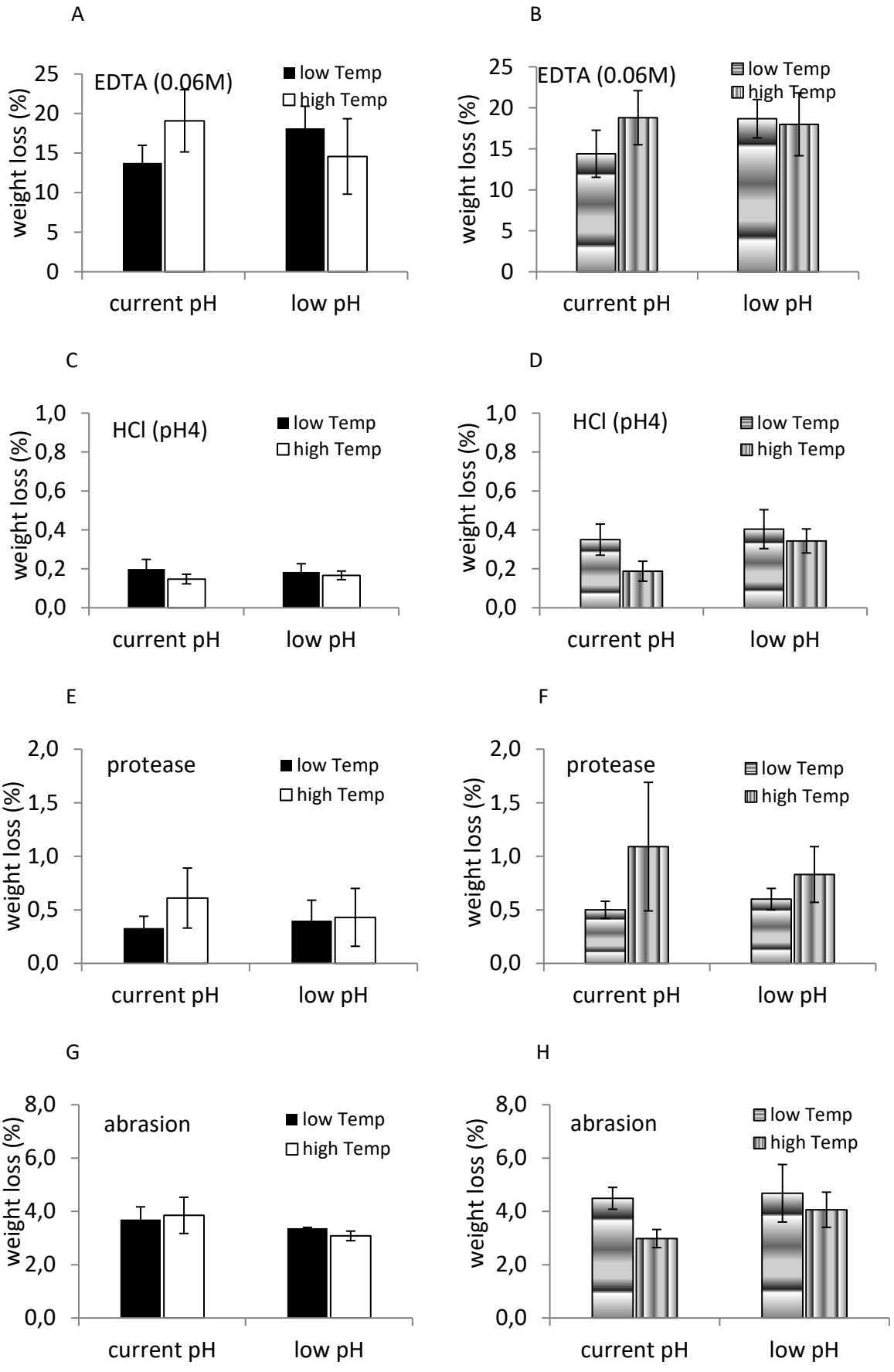
A
EDTA (0.06M)
low Temp
high Temp
weight loss (%)
25
20
15
10
5
0
current pH
low pH
B
EDTA (0.06M)
low Temp
high Temp
weight loss (%)
25
20
15
10
5
0
current pH
low pH
C
HCl (pH4)
low Temp
high Temp
weight loss (%)
1,0
0,8
0,6
0,4
0,2
0,0
current pH
low pH
D
HCl (pH4)
low Temp
high Temp
weight loss (%)
1,0
0,8
0,6
0,4
0,2
0,0
current pH
low pH
E
protease
low Temp
high Temp
weight loss (%)
2,0
1,5
1,0
0,5
0,0
current pH
low pH
F
protease
low Temp
high Temp
weight loss (%)
2,0
1,5
1,0
0,5
0,0
current pH
low pH
G
abrasion
low Temp
high Temp
weight loss (%)
8,0
6,0
4,0
2,0
0,0
current pH
low pH
H
abrasion
low Temp
high Temp
weight loss (%)
8,0
6,0
4,0
2,0
0,0
current pH
low pH

Figure 3

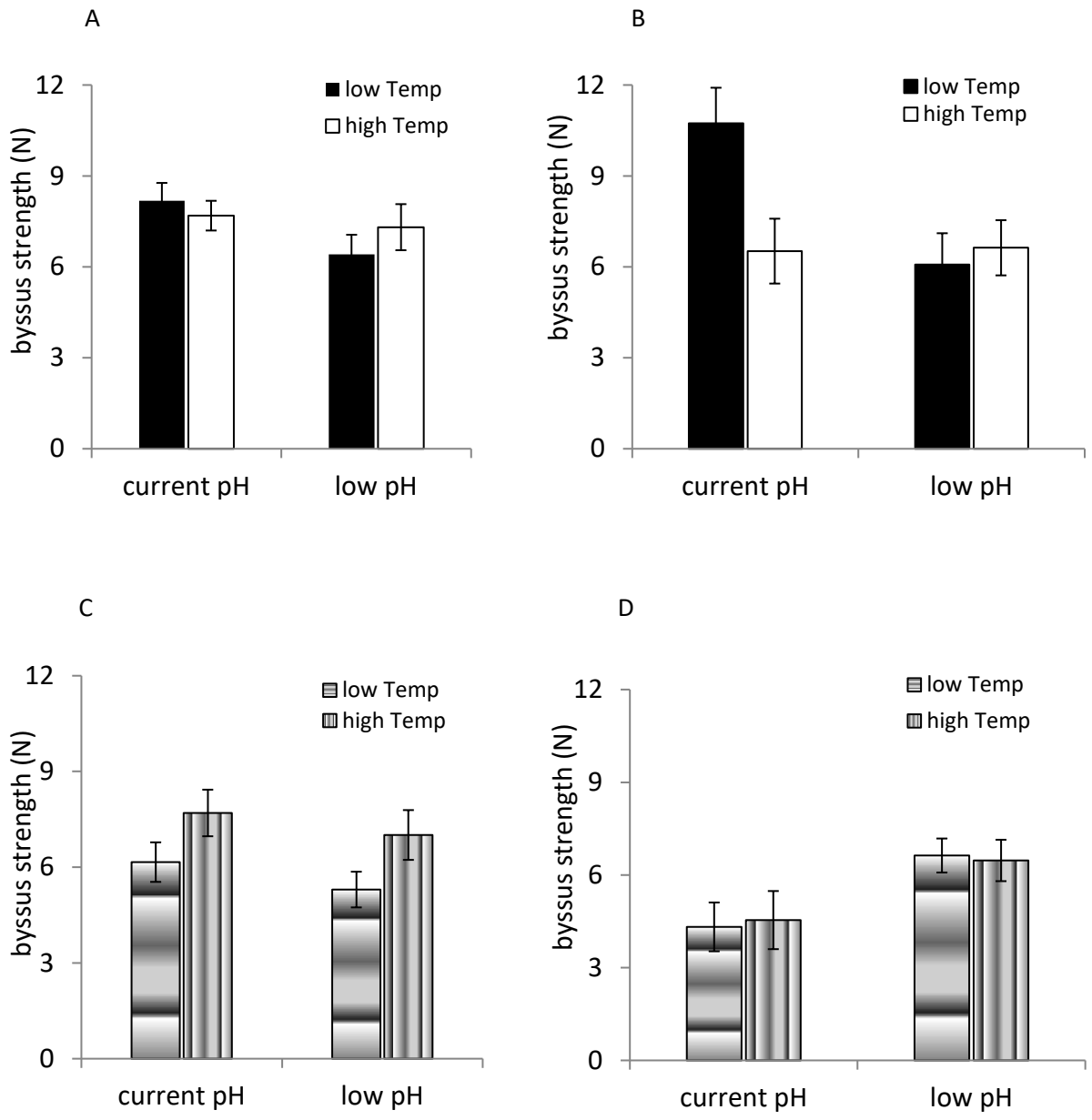
A
B
C
D
low Temp
high Temp
byssus strength (N)
0
3
6
9
12
current pH
low pH

Figure 4