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Environmental stress responses in sympatric congeneric crustaceans: Explaining and predicting the context-dependencies of invader impacts

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ABSTRACT

The role of ecophysiology in mediating marine biological pollution is poorly known. Here we explore how physiological plasticity to environmental stress can explain and predict the context-dependencies of invasive species impacts. We use the case of two sympatric skeleton shrimps, the invader *Caprella scaura* and its congener *C. equilibra*, which is currently replaced by the former on the South European coast. We compare their physiological responses to hyposalinity stress under suboptimal low and high temperature, while inferring on hypoxia tolerance. We use an energy-redox approach, analyzing mortality rate, the energetic balance and the consequent effects on the oxidative homeostasis. We found that decreased seawater salinity and/or oxygen levels can weaken biotic resistance, especially in females of *C. equilibra*, leading to periods of heightened vulnerability to invasion. Our approach provides mechanistic insights towards understanding the factors promoting invader impacts, highlighting the potential of ecophysiology for improving invasive species management.

1. Introduction

The number and frequency of non-indigenous species (NIS) introductions continue to increase, with detrimental consequences for invaded ecosystems (Pyšek et al., 2020). The synergistic effects of biological invasions with other global change drivers, such as habitat modification or global warming, make it difficult to identify the general causes of invasion success, or the causal pathways of native species decline (Didham et al., 2007). This also hampers our ability to forecast the ecological impacts of invasive species, also referred to as a form of ‘biological pollution’ (Elliott, 2003). Integrating physiology within invasion science is increasingly recognized as a powerful approach for understanding these processes and improving NIS management (Lennox et al., 2015). However, the role of physiology in mediating invasive species ecological impacts is still poorly known (Meyerson et al., 2019), especially in marine ecosystems (Lockwood and Somero, 2011). Many marine NIS inhabit human transport hubs, such as ports and marinas, where they coexist with resident species capable of living in these physiologically stressful environments (Lagos et al., 2017). Species

living in these altered habitats (commonly known as biofouling or fouling organisms) must increase their energy expenditure to successfully acclimate to environmental change and ensure cellular protection (Sokolova et al., 2012). Newcomers capable of adapting to these rapidly changing scenarios can successfully establish themselves in such environments, often reaching high abundances, overcoming previously established species and increasing their likelihood of colonizing recreational boats (Johnston et al., 2017). Species transport events through recreational boating pose additional challenges. While transported on boat hulls, fouling organisms must typically face changes in temperature, salinity and dissolved oxygen, among other factors (Davidson et al., 2008; Kauano et al., 2017). Therefore, NIS and closely-related resident species co-occurring in these altered habitats provide a unique opportunity for understanding the role of physiology in mediating marine biological invasions.

Caprellid amphipods, commonly known as skeleton shrimps, are small marine crustaceans, which are increasingly recognized a model group for understanding global change effects in marine ecosystems (e.g. Ros et al., 2014; Parretti et al., 2021). The skeleton shrimp *Caprella*

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scaura Templeton 1836 is a very aggressive caprellid species (Schulz and Alexander, 2001) and a highly efficient disperser as fouling organism on boat hulls (Krapp et al., 2006). It has become one of the most widespread NIS in European Seas since it was first found in Italy in the 1990s (Cardeccia et al., 2018). In fact, this crustacean is one of the dominant species in the biofouling communities associated to marinas along the Mediterranean Sea (Ros et al., 2015; Ulman et al., 2019). Prior to its arrival, an ecologically similar and sympatric congener, *Caprella equilibra* Say 1818, was one of the most commonly found species in Mediterranean fouling communities (Bellan-Santini and Ruffo, 1998). Interspecific competition often occurs between species occupying similar ecological niches (Pianka, 1981). In addition to sharing similar habitats (e.g. marinas), *C. scaura* and *C. equilibra* may even share similar micro-habitats (i.e. the same basibiont or biological substratum). Typical of this group of crustaceans, these species live as epibionts on a wide variety of basibionts, especially sessile organisms with an arborescent structure, such as bryozoans, hydroids or macroalgae. For example, the affinity of fouling skeleton shrimps, including *C. equilibra* and *C. scaura*, by the widespread bryozoan *Bugula neritina* it is well known (Keith, 1971; Ros et al., 2013, 2015). Moreover, these two warm-affinity congeners have similar reproductive and feeding habits and can be dispersed by similar anthropogenic transport vectors. Although *C. equilibra* is a long-term resident in Europe (e.g. Schellenberg, 1928), the origin of this widespread caprellid remains unclear. It occurs on all continents and many islands (Horton et al., 2020), in both artificial and natural habitats, and is one of the few caprellid species that can be found in planktonic samples (Takeuchi and Sawamoto, 1998). This supports a true, natural, and ancient global distribution likely achieved by both natural and anthropogenic long-distance passive dispersal. However, until its eucosmopolitan nature (sensu Darling and Carlton, 2018) is proven, we consider it as cryptogenic (sensu Carlton, 1996). A field-based study in marinas along the Iberian Peninsula (southern Europe) found that *C. scaura* is competitively displacing *C. equilibra* (Ros et al., 2015). After analyzing several environmental and demographic variables, these authors pointed out the critical role of salinity and temperature in shaping these species' distribution and modulating their interaction. Moreover, they suggest that the combined effects of lower salinity and lower temperature in the Iberian North-Atlantic coast may limit the invasion success of *C. scaura*, providing a competitive refuge for *C. equilibra*. However, while both species are known to perform well in sites with high salinity and high temperature (where they are in fact frequently observed), little is known whether these species exhibit different environmental tolerances to low salinities and/or low temperatures.

In this study, we use two sympatric skeleton shrimps (*C. scaura* and *C. equilibra*) as a model system to explore the importance of ecophysiological plasticity in explaining and predicting invader post-establishment success and context-dependencies of invader impacts. We seek causal linkages between physiological plasticity to environmental stress (such as hyposalinity under suboptimal low and high temperature, while inferring on hypoxia tolerance) in newcomer invaders and shifts in the distribution of long-term resident species. Specifically, we raise these fundamental questions: (1) does the invader (*C. scaura*) have greater physiological plasticity to cope with multiple abiotic stressors than the resident (*C. equilibra*)? (2) could these ecophysiological differences explain the observed replacement of the resident by the invader? and (3) do these differences support a further expansion of the invader to northern Europe? To answer these questions, the present study used an energy-redox approach: because facing any biological or physico-chemical stressor requires energy to fuel any necessary acclimation pathway, we analyzed the energetic balance and the consequent effects on the redox homeostasis (Rivera-Ingraham and Lignot, 2017). Particularly, we have examined the following responses of males and females of *C. scaura* and *C. equilibra* at three different levels of biological organization: (1) whole individual responses by measuring mortality rates, oxygen uptake and osmoregulatory capacity; (2) organ

responses by observing gill ultrastructure; and (3) cellular responses by measuring reactive oxidative species formation, catalases and caspases activities. Based on current distribution patterns of the two species in southern Europe (see above), we hypothesized that *C. scaura* has a broader ecophysiological plasticity than *C. equilibra* in warm scenarios, displacing the resident towards areas where the invader has not spread. Although *C. scaura* remains virtually restricted to southern Europe, the recent discovery of an isolated population in a shallow and warm estuary in the Netherlands (Faasse, 2017), leads us to hypothesize that the species could continue spreading northwards. However, we find no clear evidence to hypothesize that *C. scaura* has greater ecophysiological plasticity, and therefore greater competitiveness, than *C. equilibra* in cold scenarios.

2. Materials and methods

2.1. Animal collection

Adults of *Caprella equilibra* and *C. scaura* were collected during August 2015 from the St. Clair Marina of Sète (in the area locally known as "Basin de Cayenne"), located on the French Mediterranean Coast (43°23'46"N, 3°41'58"E). This marina was selected because it is one of the very few places in Europe where the two species occur in sympatry and with enough abundance to carry out the experiments. All arborescent substrata present in the fouling communities associated to the marina were inspected for the presence of the two species. Eventually, *C. scaura* were mostly collected from the bryozoans *Amathia verticillata* (delle Chiaje, 1822) and *Bugula neritina* (Linnaeus, 1758), while *C. equilibra* were mostly collected from the hydroid *Ectopleura crocea* (Agassiz, 1862). At the moment of collection, mean surface seawater temperature was $22.8 \pm 0.5^\circ\text{C}$ and salinity was 38.7 ± 0.1 PSU (mean \pm standard error). Animals were transported to the laboratory in cool boxes equipped with an aeration system to ensure maintaining fully oxygenated water. Acclimation to laboratory conditions lasted a minimum of 12 h to avoid handling stress before being exposed to the experimental treatments. For this, animals were sorted by sex and species and maintained in groups of a maximum of 25 caprellids in 500 ml plastic containers. These were equipped with a fine mesh serving as substrate for attachment and filled with filtered seawater at room temperature (23 °C).

2.2. Experimental setup

To compare the importance of temperature in the physiological response of the two marine congeners to low salinity stress, we selected three salinities (35, 25 and 15 PSU) and two temperatures (10°C and 25°C). Animals were therefore exposed to six experimental treatments derived from the salinity and temperature combinations, which can be found naturally in the fluctuating environments where these species live (essentially marinas and enclosed bays). Temperature selection was based on field data, which support that breeding of Mediterranean populations of *C. equilibra* and *C. scaura* are higher at intermediate temperatures (from 15 to 20 °C) and decreases at highest (~25°C) and lowest temperatures (~10°C) (Sconfietti and Lupari, 1995 and Prato et al., 2013 respectively). This allows comparison of their responses across a wide conductivity range (from normal seawater to reduced seawater salinity), under a moderated thermal stress imposed by cold and warm scenarios. We established the limit of hyposaline stress in 15 PSU because no stable populations of either species have been found under this salinity. All exposures were carried out in filtered seawater adjusted to the corresponding osmolality (determined through freezing point depression osmometry) (Model 3320, Advanced Instruments, Inc., Norwood, MA, USA) and temperature (using incubation chambers) (Sanyo incubator, model MIR 153). Exposure to such conditions lasted for 48 h, time after which mortality rates were calculated per treatment, species and sex. All survival animals were then either i) used for in-vivo

experiments (respirometry or determination of hemolymph osmotic pressure, OP) or ii) sacrificed for enzymatic analyses, imaging or quantification of oxidative stress parameters. Animals were fasted throughout the experiment to avoid the impact nutritional stress on the biomarkers considered (Herreid, 1980). Water was renewed daily and dead animals and molts were removed during this process.

2.3. Respiration measurements

Oxygen consumption rates were here determined through whole-animal respirometry for two purposes: (1) to be used as a proxy of metabolic energetic expenditure and (2) to compare the evolution of the physiological response of the two species in each treatment while decreasing the dissolved oxygen level. For this, we used 10-ml glass metabolic chambers previously equipped with an oxygen sensor spot (OXSP5, sensor code SD7-541-207, Pyro-Science GmbH, Aachen, Germany), glued to the inner side of the chamber. Each chamber was also equipped with a magnetic stirrer to ensure correct O₂ homogenization within the water column. We also included a small mesh serving as substrate for attachment to avoid the interaction of caprellids with the stirrer. Since the biomass of a single individual was not sufficient for an adequate measurement, we introduced 5 males and 5 females of one given species in the chamber for each measurement (i.e. replicate). Chambers, containing 10 ml of filtered seawater at the corresponding salinity, were then closed, ensuring the absence of any air bubbles within the chamber. Measurements were carried out at the corresponding temperature using a four-channel fiber optic oxygen meter (Firesting, Pyro-Science GmbH). All measurements started in fully oxygenated water and oxygen concentration was registered each 10 s through the Pyro Oxygen Logger software as a function of declining O₂ partial pressure (pO₂). The first 20 min of each measurement were discarded to reduce the impact of animal handling on measurements. Four measurements were recorded in parallel, in all cases one being a blank (containing no animals and serving for determining background (microbial) respiration). A total of 8 replicates were carried out for each species and experimental treatment to compare metabolic energetic expenditure. This was calculated as the linear slope of the declining O₂ trace between 90% and 70% O₂ saturation. Three of them were given enough time to consume all the oxygen within their chambers to also explore the respiratory behavior throughout pO₂ ranges and infer on hypoxia tolerance. After each measurement, all animals within the chamber were dried at 58 °C during a minimum of 48 h to calculate their dry weight (DW) and express respiration rate (RR) results, corrected for salinity and temperature, as nmol O₂·h⁻¹·mg⁻¹ DW. To estimate the respiratory behavior of these organisms as well as determine the salinity- and temperature-dependent effects on hypoxia tolerance we used two indicators: critical oxygen level, i.e. the partial pressure at which oxygen consumption begins to decrease (P_{crit}) and the regulatory index (RI). This index, proposed by (Mueller and Seymour, 2011), is a nonlinear function to estimate the ability of organisms to regulate oxygen consumption.

2.4. Osmotic pressure (OP)

OP of field and laboratory water samples were daily verified as previously described in Section 2.2. To determine the osmoregulatory behavior of *C. scaura* and *C. equilibra*, hemolymph OP was measured in eight to ten animals per treatment. To achieve this, hemolymph sample was extracted from ice-anesthetized caprellids and measured with a nano-osmometer (Clifton Osmometer, Clifton Technical physics, Hartford, NY, USA). The fusion temperature (FT) values obtained with the nano-osmometer were transformed to mOsm·kg⁻¹ using the following formula: OP = (1000 × FT) / 1.858.

2.5. Observation of gill structure

We explored potential differences in gill ultrastructure of the two species through transmission electronic microscopy (TEM) analyses. For this, dissected gills were placed for 24 h in a mixture of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1, v/v), its OP adjusted to each of the experimental conditions. Post-fixations were performed in a mixture of 1% osmium tetroxide and 0.1 M sodium cacodylate buffer (1:1, v/v) for 2 h at 4 °C. Ethanol-dehydrated samples were then embedded in Agar 100 resin (R1031, same formulation as Epon 812). Ultra-thin sections (50–150 nm) were cut on a LKB Ultratome 8800 III (LKB-Bromma, Stockholm, Sweden) or a Reichert OM2 ultramicrotome (Reichert-Jung AG, Wien, Austria) and contrasted with uranyl acetate and lead citrate prior to examination on a JEOL 1200 EX transmission electron microscope at 70 kV.

2.6. Reactive oxygen and nitrogen species formation (RONS)

We will here refer to reactive oxygen and nitrogen species (RONS) as to those molecules (radicals or not) containing one or more activated atoms of oxygen/nitrogen and are a result of their reduction. Using freshly homogenized caprellids, RONS formation was assessed following a protocol modified from Rivera-Ingraham et al. (2016). Briefly, single caprellids were freshly homogenized on ice using a manual potter and by adding 17 µl of saponin-supplemented isotonic seawater (as estimated from hemolymph OP measurements to avoid the effects of osmotic shocks on any hemolymph circulating particles). Samples were then centrifuged for 2 min at 22,000g and RONS was then fluorometrically quantified on supernatants using 5-carboxyl-2',7'-difluorodihydrofluorescein diacetate (C-H₂DFFDA, Molecular Probes C-13293, in ethanol, final concentration 10 µM). This probe is converted to its fluorescent form 2',7'-dichlorofluorescein (DCF) upon reaction with a wide variety of RONS (except superoxide anion). Since previous studies have shown a salinity-dependent fluorescence of DCF (Rivera-Ingraham et al., 2016), relative fluorescence values were normalized using a H₂O₂ curve (ranging from 0 to 0.7% H₂O₂) for each salinity treatment. Even though C-H₂DFFDA is not a H₂O₂-specific dye (e.g. Grisham, 2012), this molecule was chosen for this task for being one of the most stable, and thus abundant, RONS. All samples (in triplicates) were incubated for 10 min at 20 °C with the dye and were analyzed in a flat-bottom black microplate. Fluorescence signals (excitation: 488 nm; emission: 525 nm) were obtained with a microplate reader (Tecan Infinite M200, Tecan, Männendof, Switzerland).

2.7. Quantification of catalase and caspase activity

To conduct these analyses, a minimum of 16 samples were obtained for each species and treatment. Number of replicates depended on the number of survival animals and the extracting process success. Each replicate was composed of either 7 males or 10 females (since females are smaller than males in the two species). Animals were quickly introduced in freezing tubes, flash frozen in liquid nitrogen and stored at -80 °C until further analyses. Due to the small quantity of tissue available, one single antioxidant (catalase (CAT) activity) and one marker of cell damage (caspase 3/7 activity) were quantified for each sample. To do this, animals were weighted and homogenized on ice with a manual potter at a 1:5 ratio (w/v) using a homogenization buffer composed of 120 mM KCl 50 mM KPi (pH = 7.4) supplemented with each of the protease inhibitors aprotinin, leupeptin and aprotinin (Rivera-Ingraham et al., 2013; Strahl and Abele, 2010). Samples were centrifuged for 20 min at 13,000 rpm at 4 °C. CAT activities were measured spectrophotometrically on supernatants at 540 nm according to Johansson and Borg (1988). Results were expressed as U per mg protein, where one U of CAT activity corresponds to the formation of 1 nmol of formaldehyde per min under assay conditions. Supernatants were further diluted to achieve a final dilution of 1:50 using the

homogenization buffer and caspase 3/7 activities were measured using the Caspase Glo® 3/7 kit (Promega Corporation, Madison, WI, USA) at 20 °C and following the manufacturer's instructions. Results were expressed as relative light units (RLU) per mg protein. In all cases, values were registered in a Tecan microplate reader. Protein content of each sample was measured after Bradford (1976) and using bovine serum albumin as standard.

2.8. Data analysis

To determine the interspecific responses of the two species to the different treatments, we used a three-way univariate PERMANOVA with species, temperature and salinity as fixed factors. Additionally, to explore a possible gender effect in their intraspecific response, we use a three-way univariate PERMANOVA with temperature, salinity and sex as fixed factors for each species. Where PERMANOVA indicated a significant difference for a given factor, the source of the difference was identified using Pair-wise tests. The influence of sex was tested for all parameters except for respirometry responses (RR, RI and P_{crit}) since these parameters were measured using animal pools (each replicate consisting in 5 males and 5 females). Analyses were based on Euclidean distances matrices and statistical significance ($P < 0.05$) was tested using 9999 permutations under a reduced model. When the number or unique permutations performed was low, we use the Monte Carlo P-value instead the permutation P-value (Anderson, 2001). To estimate the P_{crit} we used both the segmented, or nonlinear 'brokenline' regression approach (Mugeo, 2003), and the 'broken-stick' regression method (Yeager and Ultsch, 1989), available in R package "RespR" (Harianto et al., 2019). We also calculated the RI using the R package "MESS" (Ekström, 2016), following the procedure implemented by Tremblay et al. (2020). They define the respiration strategies using median and quartile RI values as: low degree of oxyregulation ($0.25 < RI \text{ median} < 0.5$); high degree of oxyregulation ($0.5 < RI \text{ median} < 1$; $Q1 > 0.25$ or $Q3 > 0.75$); oxyconformity ($RI \text{ median} \sim 0$; $Q1 < 0$ and $Q3 > 0$) and metabolic suppression ($RI \text{ median}, Q1$ and $Q3 < 0$). Analyses were run in R (R Core Team, 2020) and the software package PRIMER-E v6 (Clarke and Gorley, 2006) with the PERMANOVA extension.

3. Results

3.1. Similarities in the responses of both species to the different treatments

The response of both species was similar in five of the nine parameters measured (Table 1). Thus, the respiratory rate (RR) and the regulation index (RI) did not vary significantly between species. Respiration measurements showed that for both species, an increase in temperature caused an increase in: i) RR (Figs. 1 and 2A, Table S1A); ii) oxyregulatory behavior (Fig. 2B, Table S1B) and iii) critical oxygen level (P_{crit}) (Fig. 2C, Table S1C and D). Results show a shift from metabolic depression at 10 °C to low oxyregulation at 25 °C (Table 2). However, no significant salinity-induced changes were detected for any of these parameters. The two species were osmoconformers, since the osmolality of their internal medium fluctuates according to the osmolality of the environment (Fig. 3). At the organ level, the two pairs of gills, which are attached to the ventral surface of pereonites III and IV, were sac shaped and morphologically similar in the two species (Fig. 4). In these coxal gills, the inner surface of the cuticle was covered by a monolayered epithelium (Fig. 4B). Two functional cell types were observed: thin cells (1–2 μm) with limited amount of organelles that are typical of the respiratory epithelium and thicker cells (10–20 μm), filled with numerous mitochondria flanked by apical and basal membrane infoldings (Fig. 4C–E). These cells, also named ionocytes, are classically involved in ion transport with mitochondria being functionally associated with the infoldings to facilitate ion pumping. Large patches of membranes organized as a tubulo-vesicular network were also observed along the basal side in some ionocytes. This unique organization has been

observed in the gills of the two studied caprellids kept at the different experimental salinities. At the cellular level, we did not find significant differences in the antioxidant response, measured here as catalase (CAT) activity, between the two species (Table 3C). Both produced a higher amount of CAT activity at low salinity and high temperature (Fig. 5C). We also detected a gender-specific effect on CAT activity, with females having lower levels than males in all treatments (Table 4C).

3.2. Differences in the responses of both species to the different treatments

The parameters that differed the most between the two species were P_{crit} , mortality, caspase activity and, to a lesser extent, reactive oxygen and nitrogen species (RONS) formation (Table 1). Despite the two species having similar RR and RI, the invader *C. scaura* showed a greater tolerance to hypoxia at both low and high temperatures, as indicated by a lower P_{crit} than the resident *C. equilibra* (Fig. 2C). We obtained similar results when calculating P_{crit} using the 'brokenline' and the 'broken-stick' regression method (Table S1C and D). In the two species, the highest mortality rates were reached at high temperature (25 °C) and low salinity (15 PSU) (Fig. 5A). However, low salinity treatments, at both high and low temperatures, caused a higher mortality rate in the resident species than in the invader (Fig. 5A). At high temperature, mortality rates were also higher in the resident species than in the invader at medium salinities (25 PSU) (Table 3A, Fig. 5A). In the resident species, the mortality rate decreased when salinity increased at both high and low temperature. In the invader the pattern was similar at high temperature, but there were no salinity effects at low temperature (Fig. 5A). We did not find gender-specific effects in the invader species. Conversely, we detected a higher mortality rate in females than in males of the resident species in two of the treatments: at low temperature and ambient salinity (35 PSU), and at high temperature and low salinity (15 PSU) (Table 4A).

At the cellular level, the oxidative response (RONS formation) was generally higher in the resident than in the invader species at high temperature, while the opposite pattern was found at low temperature (except for 25 PSU treatment). However, we only found clear interspecific differences in the treatment that combined low temperature and low salinity, where the resident species produced lower amount of RONS than the invader (Fig. 5B). Regarding salinity, the two species produced lower amount of RONS at reduced salinity (Table 3B, Fig. 5B). At the intraspecific level, we did not find differences in the RONS formation between treatments or sex in the invader (Table 4B). However, an interactive effect of gender with salinity and temperature was detected in the resident species, with females producing higher amount of RONS than males at oceanic salinity (35 PSU), at both high and low temperatures. Regarding cellular damage, caspases activities were significantly higher in the resident species than in the invader (Table 3D, and Fig. 5D). Differences were significant in all treatments except for 25 PSU and 25 °C. In the two species, caspases activities were influenced by the interactive effect of salinity and temperature (Table 4D), and the highest caspase activity was recorded for the treatment of 15 PSU and 10 °C (Fig. 5D). Differences between genders were only found for *C. scaura*, where females showed a higher amount of caspases activities than males (Table 4D).

4. Discussion

One of the major challenges to forecast how a species range may change in response to the spread of a non-indigenous species is to identify the processes or mechanisms underlying this ecological impact. Integrating physiology within invasion ecology we provide mechanistic insights towards understanding the displacement of a long-term resident crustacean, *Caprella equilibra*, by an introduced congener, *C. scaura*, in changing environments. Our study shows that while the two species display a similar ecophysiological performance at both cold (10 °C) and warm temperatures (25 °C) in oceanic seawater (35 PSU), the invader

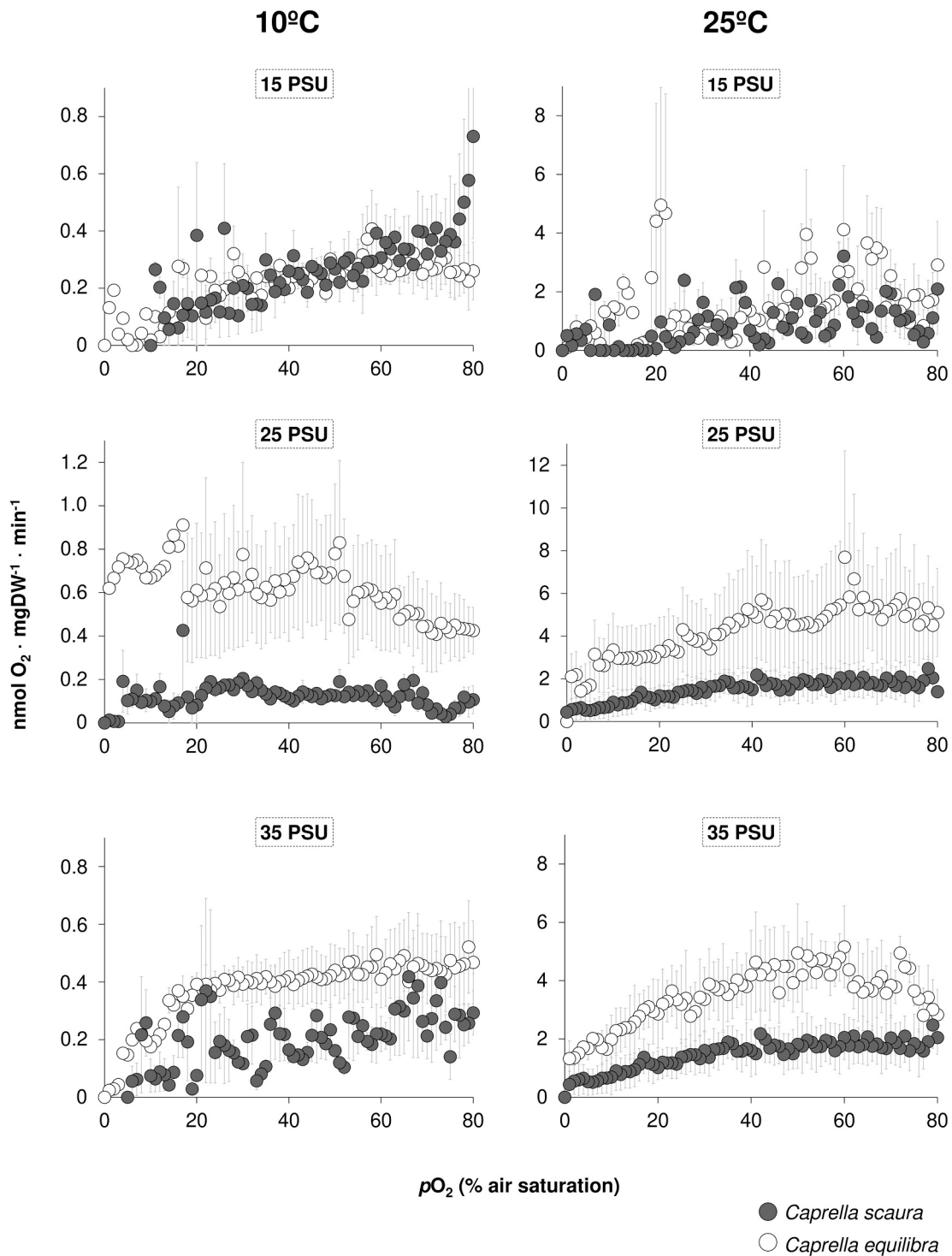


Fig. 1. Whole animal respirometry of the resident species (*C. equilibra*) and the invader (*C. scaura*) in the different treatments. Error bars correspond to the standard error among replicates (each replicate consisting in 10 individuals pooled) and the units are given per weight.

showed a higher ecophysiological plasticity than the resident to deal with reduced salinity and hypoxic stress. We discuss here how these findings may explain and predict the context-dependencies of the invader post-establishment success and impacts. This is the first time that an energy-redox approach is applied in skeleton shrimps, revealing new and interesting information on the autecology of these understudied taxa.

4.1. On the ecophysiology of skeleton shrimps: does the invader have greater physiological plasticity to cope with multiple abiotic stressors than the resident?

While several studies have addressed the environmental tolerance of both introduced and native skeleton shrimps (e.g. Cockman and Albone, 1987; Takeuchi et al., 2003; Ashton et al., 2007), little is known on the ecophysiological background underpinning this tolerance. We show that both *Caprella scaura* and *C. equilibra* are osmoconformer species with a

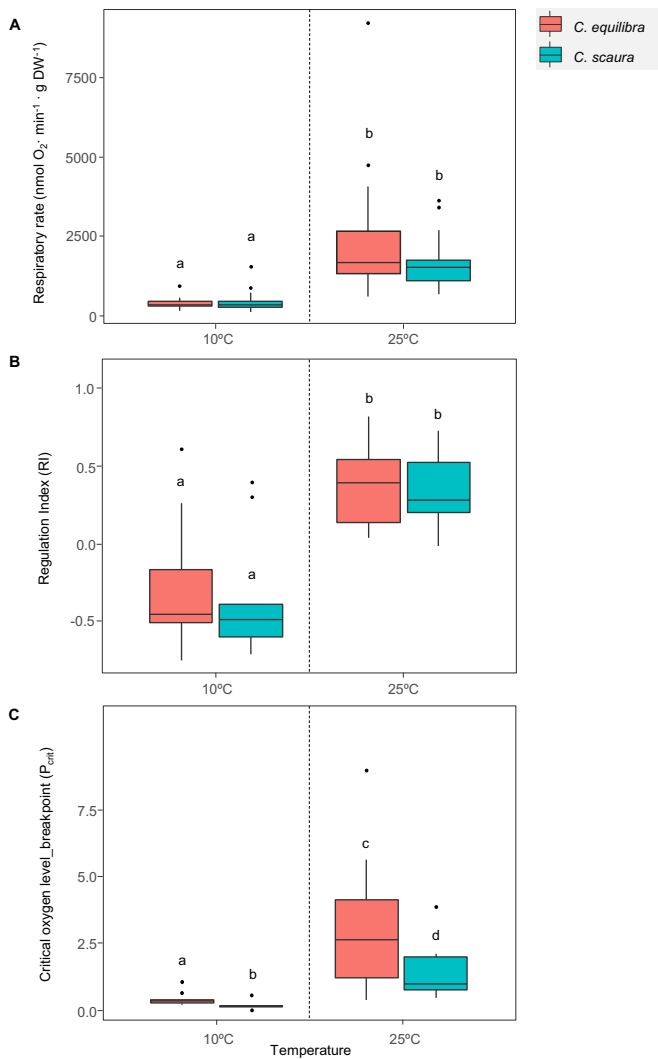


Fig. 2. Comparative values of the respiratory rate (A), regulation index (B), and critical oxygen level (C) in the two species at 10 °C and 25 °C. Significant differences ($P < 0.05$) between species were presented with different letters (results of PERMANOVA analysis can be found in Table S1).

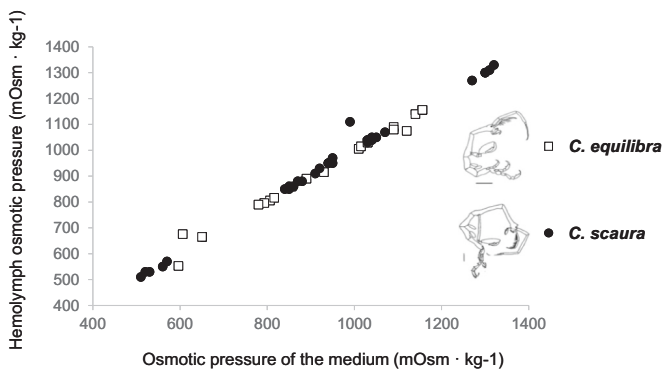


Fig. 3. Osmolality curves in the two species. Bars in the caprellids lateral view figures indicate 1 mm.

similar gill ultrastructure. Therefore, when exposed to low salinities, they do not invest energy in cellular transport mechanisms to regulate internal osmolality (Lignot and Charmantier, 2015). This may explain in part why the exposure to different salinities had no significant effects on

their metabolic energy expenditure (measured as respiratory rates). It may also explain why caprellids, compared to other amphipods, are mostly stenohaline marine invertebrates, which are typically the case for osmoconformers (Lignot and Charmantier, 2015). However, low salinity effects were higher in the resident than in the invader species at both the individual (i.e. mortality rates) and cellular (i.e. caspase activity) levels, pointing to a higher tolerance to hyposalinity stress in the invader. This agrees with Cockman and Albone (1987) who found a wider salinity tolerance in *C. scaura* than in *C. equilibra* in coexisting populations of the River Swan Estuary (Australia). They also found that *C. equilibra* was limited to high salinity regions of the estuary, while *C. scaura* penetrated into brackish water areas. Sconfiatti and Lupari (1995) found that *C. equilibra* populations in a Venetian lagoon (Italy) were able to tolerate cyclic decreases of salinity values down to 15 PSU, but only reported stable populations from higher salinities. The hyposalinity tolerance was modulated by seawater temperature in the two species, but this effect varied depending on the species and the physiological response analyzed. At the individual level, the two species tolerate a wider range of salinities in cold than in warm scenarios, as indicated by their survival rates. However, while low salinity reduced the survival rate of *C. equilibra* at both low and high temperatures, in the invader it only decreased at high temperature (and to a lesser extent than in *C. equilibra*). At the cellular level, reduced salinity triggered a significantly high antioxidant response (i.e. catalase activity) in the two species, but especially at high temperature. This protective cellular response was probably not enough to buffer the effect of reduced salinity in cellular homeostasis at low temperature, as indicated by the higher levels of cell damage (i.e. caspase activity) found at 15 PSU and 10 °C (especially in *C. equilibra*). Considering that metabolic energetic expenditure significantly decreased with decreasing temperature in the two species, we hypothesize that hyposalinity effects take longer to act at individual level at 10°C than at 25°C. Anyway, these cellular markers would be a warning, for example, that the invader also suffers from hyposalinity stress in cold scenarios, despite the absence of evidence at the individual level (i.e. mortality rate and RR). This highlights the importance of using a multilevel approach for addressing the effects of environmental stress on physiological systems (Somero, 2012). This approach also reflects that the effects of hyposalinity stress on cellular homeostasis and survival rate is higher in the resident than in the invader crustacean under both cold and warm scenarios. We did not find clear evidence that these individual and cellular responses to hyposaline stress are RONS (reactive oxygen and nitrogen species)-mediated.

In addition to a higher tolerance to reduced salinity, we also found that *C. scaura* was less sensitive to hypoxic stress than *C. equilibra*, as reflected by the lower values of critical oxygen level (P_{crit}) at both high and low temperature. The hypoxic sensitivity of *C. equilibra* was already observed in the field by Sconfiatti and Lupari (1995). They found that the high temperatures of midsummer (i.e. 25°C) coupled with low dissolved oxygen dampened both the growth and breeding of this species. As occurred with hyposalinity tolerance, the hypoxia tolerance and the oxyregulatory behavior was clearly influenced by the temperature in the two species. Both reduce their tolerance to hypoxia at high temperatures as the metabolic demand increases. Moreover, both shift from a low oxyregulatory strategy in warm scenarios to a metabolic depression in cold scenarios, as indicated by the RI values. This last strategy, which entails the suppression of total energy consumption by limiting processes that demand high energy expenditure (Seibel, 2011), is a common response among marine animals to environmental stressors (Storey and Storey, 1990). It has been associated to both hypoxia-sensitive (Alexander and McMahon, 2004) and hypoxia-tolerant species (Seibel et al., 2016; Tremblay et al., 2020). For example, hyperiid amphipods go into metabolic depression to survive in the hypoxic and cold deep sea waters when migrating in the water column (Seibel et al., 2016). However, to successfully recover from hypoxic periods they must have effective mechanisms to minimize oxidative stress during hypoxia-reoxygenation transgression (Hermes-Lima et al., 2015). This, as well as the

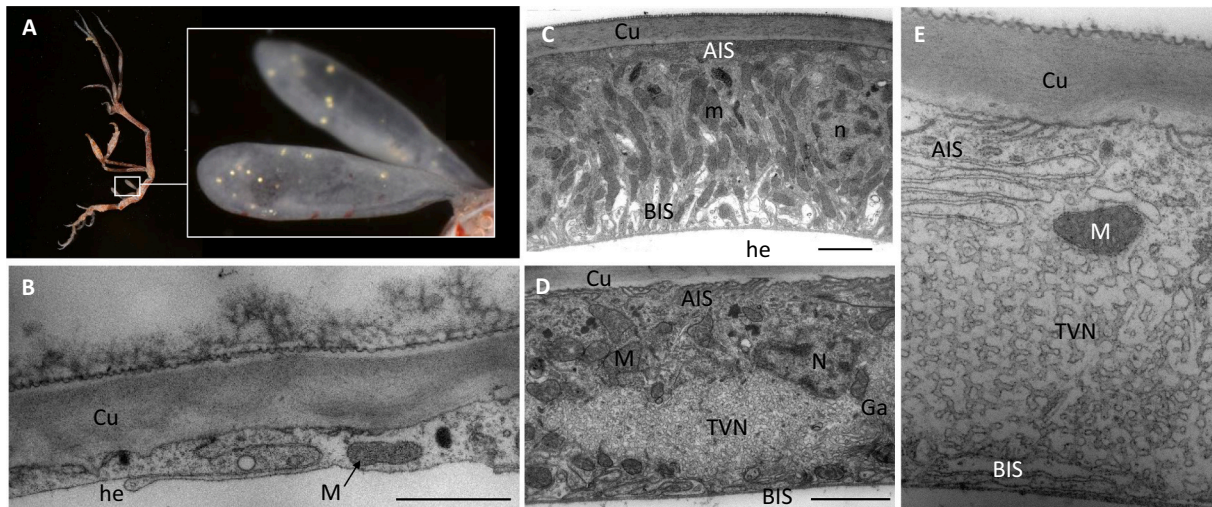


Fig. 4. Electron microscope pictures of the gill epithelia of *Caprella scaura* and *C. equilibra*. A) Coxal gills of *C. scaura*, B) respiratory epithelium of *C. scaura*, C) ion-transporting cells (ionocytes) of *C. scaura*, D, E) ion-transporting cells of *C. equilibra*. Note in these cells, the basal infoldings projecting deeply into the cytoplasm and the numerous mitochondria. In some ionocytes, a dense network of membranes can be observed. AIS: apical infolding system, BIS: basolateral infolding system, Cu: cuticle; He: hemocoel, M: mitochondria, Nu: nucleus; TVN: tubulo-vesicular network. Bars indicate 1 μm .

relationship between negative RI values and metabolic depression, remains to be confirmed in caprellid amphipods. Moreover, as the animals were collected during summer they were not acclimatized to cold temperatures. It would be interesting to carry out the same experiments on coexisting populations of the two species collected in winter or in colder areas. However, the large number required for the experiments could be a limiting factor.

Several authors have found differences in environmental tolerance between female and male amphipods in general (e.g. [Correia et al., 2003](#), [Sornom et al., 2010](#), [Bedulina et al., 2017](#)) and in caprellids in particular (e.g. [Ashton et al., 2007](#); [Ros et al., 2014](#); [Parretti et al., 2021](#)). Although we found that species-related differences were more evident than sex-related differences in physiological tolerance, we observed gender-effects in some treatments reflecting the importance of considering this factor for understanding the stress response in these species. For example, the catalase activity was lower in females than in males, independent of the treatment or the species. This difference, which has also been observed in other amphipods ([Correia et al., 2003](#)), may imply a lower redox buffering capacity in females. Thus, in *C. equilibra*, mortality rates and oxidative responses (i.e. RONS formation) were higher in females than in males in some of the treatments. In *C. scaura*, we observed higher caspase activity in females than males. Overall, this may reflect a slightly higher susceptibility of females to environmental stress and/or a different way of dealing with stress from a physiological point of view. However, more studies are needed to be able to draw clear conclusions about inter-sex differences in susceptibility to stress in these species. Since we only use adults, further studies are also needed to investigate potential inter-age differences.

In summary, despite the two species being osmoconformers and sharing a similar gill ultrastructure, *C. scaura* is less sensitive to hypo-salinity stress than *C. equilibra*, which suffers higher degrees of cellular damage and mortality rates. Moreover, while the two species have similar rates of oxygen consumption, *C. scaura* showed a greater tolerance to hypoxia, as indicated by a lower critical oxygen partial pressure than *C. equilibra*. The increased ability of the invader to cope with acute drops in salinity and oxygen occurred both at low and high temperatures.

4.2. Could these physiological differences explain the observed replacement of the long-term resident by the invader?

A wide physiological tolerance increases the advantage in competing with sympatric species in the invaded ecosystem ([Ricciardi and Rasmussen, 1998](#)). Overall, our findings indicate that, while the two species can coexist in sympatry in cold and warm environments, the invader likely overcomes the resident congener in stressful periods where salinity and oxygen levels drop (as it was discussed above). While temperature plays an important role, interacting with the aforementioned abiotic factors, it seems that by itself it is not a decisive factor explaining the current replacement of *C. equilibra* by *C. scaura*. In fact, [Cockman and Albone \(1987\)](#) did not find significant differences in the upper and lower thermal tolerance between two coexisting populations of these species. [Parretti et al. \(2021\)](#) recently explored the biotic interaction between the two species in a future climate change scenario for Madeira Island (with a seawater temperature of 29°C). They found that survivorship or interspecific displacement of *C. scaura* and *C. equilibra* will not be compromised when they coexist in similar abundances at this temperature. However, the synergistic effects of high temperature and higher abundance of *C. scaura* than *C. equilibra* triggered the displacement of the resident (while it did not affect their survival). Our findings suggest that dominance for *C. scaura* over *C. equilibra* could be achieved in presence of hyposalinity and/or hypoxic stress. Specifically, the synergistic effect of both stressors may allow the ecological dominance of the invader by reducing the competitive ability of the resident species. Competition between species represents a key form to resistance post establishment invasion ([Kimbrow et al., 2013](#)). Therefore, decreased competitiveness may in turn weaken biotic resistance to invaders ([Liancourt et al., 2005](#)). Competition is a context-dependent process which can be regulated by stress levels (as discussed above), but also by resources ([McQuaid et al., 2015](#)). The two species coexist in marinas, highly disturbed habitats where resources such as food and space are usually abundant. Within these habitats, *C. scaura* and *C. equilibra* can also share similar basibionts, such as the bryozoan *Bugula neritina* (see [Introduction](#)). However, not all basibionts, even if they belong to the same species, offer the same habitability resources for them ([Ros et al., 2013](#)). This will depend on several factors, such as those related to their structural complexity, size, color, healthiness or location, affecting the epibiont choice (e.g. [Martínez-Laiz et al., 2018](#)). If *C. scaura* and *C. equilibra* coexist in the same colony of

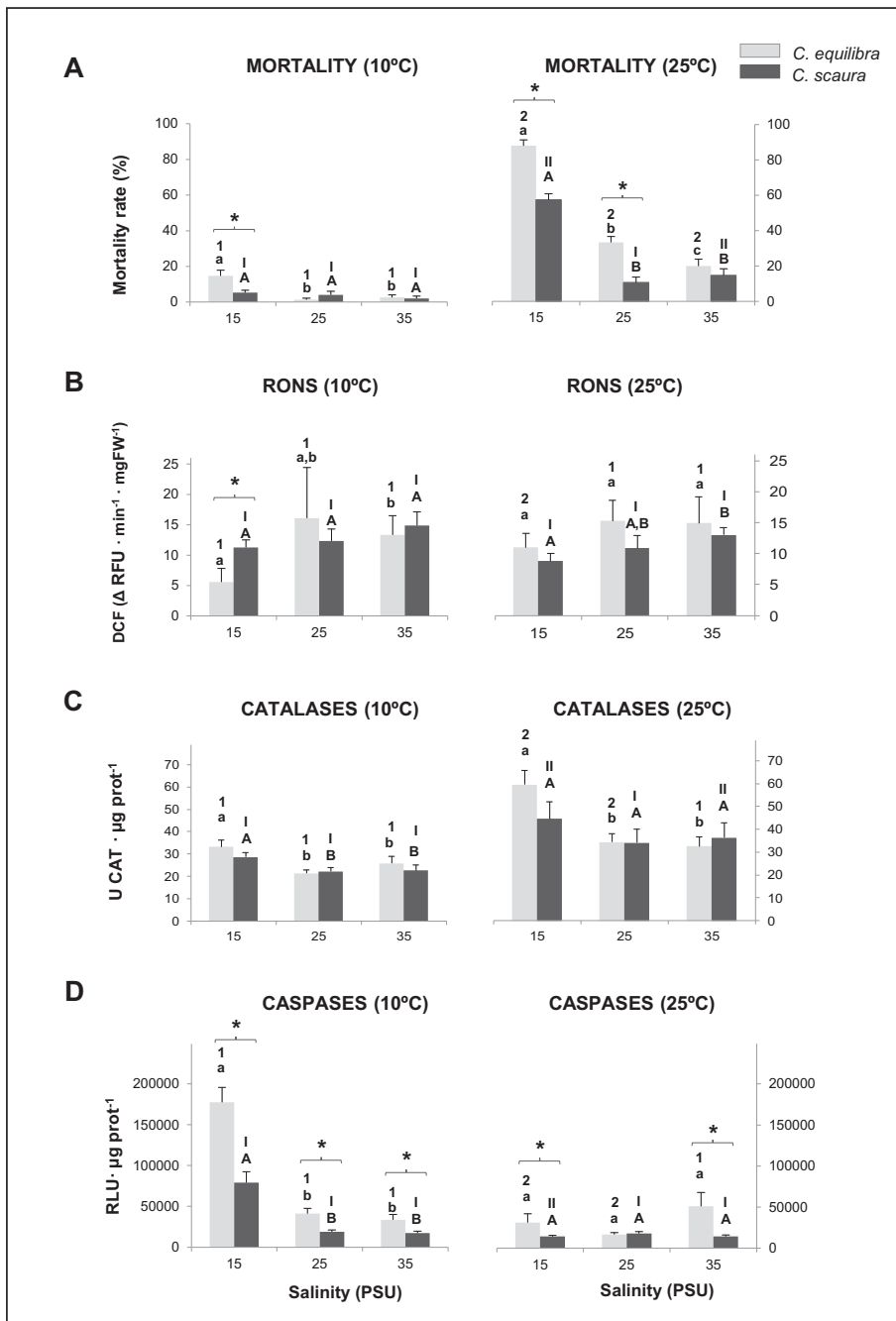


Fig. 5. Pair-wise results of the interaction species × temperature × salinity showing the intraspecific and interspecific differences in mortality rates (A), reactive oxygen and nitrogen species (RONS) production (B), catalase activity (C) and caspases activity (D) of *C. scaura* and *C. equilibra* individuals among salinity and temperature combinations. Interspecific significance differences ($P < 0.05$) among salinities (15, 25 and 35 PSU) per each level of temperature (10°C and 25°C) were presented with different letters (lowercase letters for *C. equilibra* and uppercase letters for *C. scaura*). Significant differences between temperatures per each level of salinity were presented with different numbers (cardinal numbers for *C. equilibra* and roman numbers for *C. scaura*). Significant differences ($P < 0.05$) between species, at each salinity and temperature combination, were presented with asterisks.

B. neritina, they can compete for a shared space. Following the principle of competitive exclusion (Hutchinson, 1965), when this type of ‘high quality substratum’ is limited and becomes overcrowded, a strong interspecific competition may occur (Pianka, 1981). This may lead to spatial resource partitioning (Schoener, 1974), with caprellids occupying different substrata. This pattern has been observed in coexisting populations of *C. equilibra* and *C. scaura* in distant regions, such as Australia (Cockman and Albone, 1987), Spain (Guerra-García et al., 2011; Ros et al., 2013, 2014) and even the study area (pers. obs.). These authors describe how *C. equilibra* is mostly marginalized to hydroids in marinas where arborescent bryozoans are dominated by *C. scaura*. However, in the absence of the invader, or when its abundance is very low (as occurs close to its non-native distribution limits), *C. equilibra* is frequently found in high abundances in these bryozoans (e.g. Ros et al., 2014; Guerra-García et al., 2015). As discussed above, hyposalinity and/

or hypoxic stress would decrease the energy budget for competition in *C. equilibra*, offering a window of opportunity to the invader to become the dominant species. This may lead to a process of habitat segregation, where *C. equilibra* is displaced towards poorer quality substrata where the invader is absent. A low quality microhabitat, coupled with the aforementioned abiotic stressors, can further compromise its survival in a particular location. Therefore, environmental stress can modulate the biotic interaction between these species by affecting them directly, through changes in their ecophysiological response, but also indirectly, by reducing or enlarging their micro-spatial niche (i.e. basibiont availability). It is noteworthy that processes other than resource competition may result in similar exclusion patterns, especially at local scales. For example, a disturbance causing the removal of *C. equilibra* (as a result of maintenance work in the marina, cleaning processes, etc.) may facilitate the establishment of a subsequently introduced population of *C. scaura*.

Table 1

Summary of the comparative responses between the resident species (*C. equilibra*) and the invader (*C. scaura*) to the different treatments. Interspecific differences are highlighted in red. I: invader, R: resident, RR: respiratory rate, RI: regulation index, P_{crit}: critical oxygen level, RONS: reactive oxygen and nitrogen species.

Treatment		Parameters measured at different levels of biological organization								
		Mortality rate	Individual			Osmoregulatory capacity	Organ ultrastructure	Cellular		
			RR	RI	P _{crit}			RONS	Catalases	Caspases
T ^a (°C)	Salinity (PSU)									
10	15	I < R	I = R	I = R	I < R	I = R	I = R	I > R	I = R	I < R
	25	I = R	I = R	I = R	I < R	I = R	I = R	I = R	I = R	I < R
	35	I = R	I = R	I = R	I < R	I = R	I = R	I = R	I = R	I < R
25	15	I < R	I = R	I = R	I < R	I = R	I = R	I = R	I = R	I < R
	25	I < R	I = R	I = R	I < R	I = R	I = R	I = R	I = R	I = R
	35	I = R	I = R	I = R	I < R	I = R	I = R	I = R	I = R	I < R

Table 2

Regulation index values for each species at 10 °C and 25 °C.

Species	T (°C)	RI			Strategy
		Median	Q1	Q3	
<i>C. equilibra</i>	10	-0.46	-0.51	-0.17	Metabolic depression
	25	0.40	0.14	0.55	Low oxyregulation
<i>C. scaura</i>	10	-0.50	-0.61	-0.39	Metabolic depression
	25	0.29	0.21	0.53	Low oxyregulation

However, at a higher spatial scale, competitive exclusion is more likely responsible for the observed patterns.

4.3. Can we expect a further expansion of the invader to northern Europe?

We think that the further spread of *C. scaura* along northern European regions is highly probable, at least in regions with seawater temperatures up to 10°C, such as the Iberian North-Atlantic coast. We

suggest this considering that: (1) its ecophysiological plasticity in cold scenarios is higher in comparison with other congeners that are well established in this area, such as *C. equilibra*; (2), the Atlantic populations of *C. scaura* are more recent than the Mediterranean ones (Cabezas et al., 2014), suggesting that the species is in a phase of active colonization in this direction; and (3), its ability for anthropogenic transportation, increasing its chances of spreading beyond its current distribution limit. Likewise, the presence of an isolated population in the Netherlands may imply that the species is already present in several areas of northern Europe where it remains unnoticed. Moreover, we forecast a further displacement of *C. equilibra* in these new invaded locations, especially in periods of hypoxia and hyposalinity. However, it is worth mentioning that in marinas of the North-Atlantic coast of Europe, *C. scaura* will have to face a cold-adapted and bigger invasive congener, the Japanese skeleton shrimp *Caprella mutica* Schurin, 1935. This species has been related with the displacement of other congeners (Shucksmith et al., 2009). While *C. mutica* does not survive in salinities lower than 16 PSU, it is able to survive at temperatures as low as -1.8°C (Ashton et al., 2007). Further studies would be necessary to accurately predict the

Table 3

Results of the PERMANOVA analyse to test interspecific differences for each salinity and temperature combination in the mortality rate (A), reactive oxygen and nitrogen species (RONS) production (B), catalase activity (C) and caspases activity (D). Pair-wise tests for the factors species, temperature and salinity are represented in Fig. 1.

Source of deviation	A) Mortality rate				B) DCF fluorescence (RONS formation)			
	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Species = Sp	1	2178.00	45.84	0.0001***	1	1.17	2.77	0.1030
Temperature = Te	1	18,818.00	396.08	0.0001***	1	0.34	0.79	0.3756
Salinity = Sa	2	7358.00	154.87	0.0001***	2	1.95	4.59	0.0133*
Sp × Te	1	1317.60	27.73	0.0001***	1	2.10	4.96	0.0278*
Sp × Sa	2	438.00	9.22	0.0006***	2	0.37	0.88	0.4188
Te × Sa	2	4168.70	87.74	0.0001***	2	0.21	0.50	0.6052
Sp × Te × Sa	2	180.22	3.79	0.0274*	2	0.51	1.21	0.3085
Residual	60	47.51			108	0.42		
Transformation		None				Log (X + 1)		

Source of deviation	C) Catalase activity				D) Caspase 3/7 activity			
	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Species = Sp	1	0.69	3.81	0.0531	1	13.92	32.93	0.0001***
Temperature = Te	1	5.64	31.29	0.0001***	1	22.04	52.15	0.0001***
Salinity = Sa	2	2.38	13.21	0.0001***	2	11.00	26.04	0.0001***
Sp × Te	1	0.08	0.43	0.5197	1	0.83	1.97	0.1624
Sp × Sa	2	0.20	1.10	0.3382	2	0.69	1.62	0.2028
Te × Sa	2	0.08	1.47	0.6238	2	10.69	25.28	0.0001***
Sp × Te × Sa	2	0.18	0.99	0.3735	2	1.34	3.16	0.0434*
Residual	175	0.18			171	0.42		
Transformation		Log (X + 1)				Log (X + 1)		

* P < 0.05.
 ** P < 0.01.
 *** P < 0.001.

Table 4

Results of the PERMANOVA analyse to test the gender effect in the intraspecific differences for each salinity and temperature combinations in the mortality rate (A), reactive oxygen and nitrogen species (RONS) production (B), catalase activity (C) and caspase activity (D).

A)	<i>Caprella equilibra</i>				<i>Caprella scaura</i>			
	Source of deviation	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F
Temperature = Te	1	1504.70	403.05	0.0001***	1	5088.40	120.52	0.0001***
Salinity = Sa	2	5584.00	149.57	0.0001***	2	2212.00	52.39	0.0001***
Sex	1	1.78	0.05	0.8306	1	100.00	2.37	0.138
Te × Sa	2	2529.80	67.76	0.0001***	2		43.08	0.0001***
Te × Sex	1	44.44	1.19	0.2861	1	75.11	1.78	0.1985
Sa × Sex	2	23.11	0.62	0.5494	2	25.33	0.60	0.5596
Te × Sa × Sex	2	268.44	7.19	0.0033**	2	43.11	1.02	0.3704
Residual	24	37.33			24	42.22		
Transformation			None				None	
Pair-wise test (Sex):			<i>Te × Sa (Sex) 10°C and 35 psu: f>m</i> <i>25°C and 15 psu: f>m</i>				-	
B)			DCF Fluorescence (RONS)				DCF Fluorescence (RONS)	
Source of deviation	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Temperature = Te	1	2.33	4.86	0.0291*	1	0.54	2.80	0.1011
Salinity = Sa	2	1.48	3.08	0.0521	2	0.59	3.07	0.0547
Sex	1	3.16	6.59	0.0095**	1	0.32	1.67	0.2052
Te × Sa	2	0.84	1.76	0.1836	2	0.06	0.32	0.7234
Te × Sex	1	0.41	0.86	0.3585	1	0.06	0.30	0.5748
Sa × Sex	2	2.09	4.36	0.0176*	2	0.32	1.67	0.1983
Te × Sa × Sex	2	2.19	4.56	0.0135*	2	0.17	0.90	0.4078
Residual	48	0.48			48	0.19		
Transformation			Log (X + 1)				Log (X + 1)	
Pair-wise test (Sex):			<i>Te × Sa (Sex) 10°C and 35 psu: f>m</i> <i>25°C and 35 psu: f>m</i>				-	
C)			Catalase activity				Catalase 3/7 activity	
Source of deviation	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Temperature = Te	1	2.60	28.42	0.0001***	1	2.05	16.95	0.0003***
Salinity = Sa	2	1.53	16.73	0.0001***	2	0.88	7.26	0.0018**
Sex	1	2.00	21.91	0.0001***	1	9.34	77.05	0.0001***
Te × Sa	2	0.15	1.61	0.2085	2	0.06	0.50	0.6055
Te × Sex	1	0.10	1.09	0.3017	1	1.07	8.80	0.0033**
Sa × Sex	2	0.06	0.68	0.5119	2	0.39	3.21	0.0451*
Te × Sa × Sex	2	0.09	0.93	0.3985	2	0.09	0.74	0.4833
Residual	73	0.09			90	0.12		
Transformation			Log (X + 1)				Log (X + 1)	
Pair-wise test (Sex):			<i>(Sex) f<m</i>				<i>Te (Sex) 10°C: f<m; 25°C: f<<m</i> <i>Te (Sex) Sa (Sex) 15 psu: f<m; 25 psu: f<<m; 35 psu: f<<m</i>	
D)			Caspase activity				Caspase 3/7 activity	
Source of deviation	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Temperature = Te	1	13.10	22.71	0.0001***	1	9.01	42.90	0.0001***
Salinity = Sa	2	5.36	9.30	0.0004***	2	4.92	23.45	0.0001***
Sex	1	0.82	1.43	0.2341	1	8.78	41.78	0.0001***
Te × Sa	2	5.34	9.27	0.0002***	2	5.69	27.09	0.0001***
Te × Sex	1	0.45	0.78	0.3764	1	0.55	2.64	0.1091
Sa × Sex	2	0.90	0.78	0.4551	2	0.21	1.00	0.3800
Te × Sa × Sex	2	0.26	0.23	0.7937	2	0.24	1.16	0.3195
Residual	70	40.36			89	0.21		
Transformation			Log (X + 1)				Log (X + 1)	
Pair-wise test (Sex):			-				<i>(Sex): f>m</i>	

*P < 0.05.
**P < 0.01.
***P < 0.001.

outcome of the potential interaction between these two invaders. Global change may involve increases in temperature but also in precipitation, leading to increasing hyposalinity stress (Somero, 2012). The high ability of *C. scaura* to deal with short-term hyposalinity periods may enhance its success and competitiveness as an invader, but also its ability to cope with global change effects. Considering that marinas can receive considerable freshwater inputs, physiological adaptations to low salinities may also be determinant for a further spread of the species across these anthropogenic habitats. Moreover, hypoxia in marine and estuarine environments is increasing worldwide primarily due to anthropogenic causes (Diaz and Rosenberg, 2008). Jewett et al. (2005) found that moderately low levels of dissolved oxygen enhance success of

invasive and cryptogenic epifaunal species. This stressor is particularly important in water bodies with restricted water circulation due to anthropogenic structures, such as marinas (Lagos et al., 2017). Therefore, adaptations to hypoxia may be determinant for the establishment success of *C. scaura* in marinas. Moreover, species with high ability to cope with hypoxia could overcome low or changing oxygen conditions during transportation. This may occur, for example, during ballast water transport (Verberk et al., 2018) and when colonizing boat hulls, ropes or buoys which are temporary removed from the water.

5. Conclusions

Despite the increasing concern about the impact of marine invasions, we still understand little about the factors promoting these processes. Using comparative ecophysiology, our study sheds light on the underlying mechanisms explaining the almost complete exclusion of *C. equilibra* detected in many locations of southern Europe now dominated by *C. scaura*. We found that the invader has a higher tolerance to hyposalinity and hypoxic stress than the resident species, in both cold and warm scenarios. Therefore, while the two species can coexist in sympatry in cold (10°C) and warm (25°C) environments, decreased seawater salinity and/or oxygen levels may lead to periods of heightened vulnerability to invasion. Anthropogenic activities are increasing the frequency and extent of sudden environmental changes, challenging the ecophysiological limits of species (Rivera-Ingraham and Lignot, 2017). This may ultimately explain the replacement of long-term resident species inhabiting marinas, which are supposedly well pre-adapted to anthropogenic pressures, by newcomer invaders which are better adapted to acute multi-stressor changes. Therefore, an increase in species turnover in highly invaded habitats may be an indicator of the accelerated rate of anthropogenic global change.

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CRedit authorship contribution statement

Macarena Ros: Conceptualization, Sampling, Methodology, Formal analysis, Investigation, Writing – Original draft, Review and editing. José Manuel Guerra-García: Conceptualization, Sampling, Methodology, Investigation, Funding acquisition, Writing - Reviewing and editing. Jehan-Hervé Lignot: Conceptualization, Sampling, Methodology, Investigation, Resources, Writing – Reviewing and editing. Georgina A. Rivera-Ingraham: Conceptualization, Sampling, Methodology, Investigation, Resources, Funding acquisition, Writing – Reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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