**Title: METABOLIC RESPONSES TO TEMPERATURE STRESS UNDER elevated *p*CO2 IN THE SLIPPER LIMPET *Crepidula FORNICATA***

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**Short running head:** *C. fornicata* respiration under high *p*CO2

**ABSTRACT**

In the current context of environmental change, ocean acidification is predicted to affect the cellular processes, physiology and behavior of all marine organisms, impacting survival, growth and reproduction. In relation to thermal tolerance limits, the effects of elevated *p*CO2 could be expected to be more pronounced at the upper limits of the thermal tolerance window.

Our study focused on *Crepidula fornicata*, an invasive gastropod which colonized shallow waters around European coasts during the 20th century. We investigated the effects of 10 weeks’ exposure to current (380 µatm) and elevated (550, 750, 1000 µatm) *p*CO2 on this engineer species using an acute temperature increase (1°C 12h-1) as the test. Respiration rates were measured on both males (small individuals) and females (large individuals). Mortality increased suddenly from 34°C, particularly in females. Respiration rate in *C. fornicata* increased linearly with temperature between 18°C and 34°C, but no differences were detected between the different *p*CO2 conditions either in the regressions between respiration rate and temperature, or in *Q*10 values. In the same way, condition indices were similar in all the *p*CO2 treatments at the end of the experiment but decreased from the beginning of the experiment. This species was highly resistant to acute exposure to high temperature regardless of *p*CO2 levels, even though food was limited during the experiment. *C. fornicata* appears to have either developed resistance mechanisms or a strong phenotypic plasticity to deal with fluctuations of physico-chemical parameters in their habitat. This suggests that this invasive species may be more resistant to future environmental changes compared to its native competitors.

**Keywords**: CO2 stress, invasive species, ocean acidification, *Q*10, respiration, temperate waters

**INTRODUCTION**

As part of global change, ocean acidification is caused by increasing anthropogenic CO2 emissions which have increased since the beginning of the industrial revolution (Solomon *et al.,* 2007). Future *p*CO2 increases are predicted to reduce the pH of surface waters by 0.3 - 0.4 units by the end of the century (Caldeira & Wickett, 2003). Such decreases will produce changes in carbon and carbonate seawater chemistry through decreased carbonate ion concentrations (CO32-) and a lower calcium carbonate saturation state (Ω). These changes are predicted to have major consequences for marine life (Fabry *et al.*, 2008; Kroeker *et al.*, 2013b) and, especially, could have broad impacts on physiological functions of heterotrophic marine organisms (Pörtner, 2008; Hofmann & Todgham, 2010).

The decrease in pH is likely to have a wide range of effects on marine invertebrates via shifts in acid-base homeostasis, changes in metabolism and energy balance (Pörtner *et al.*, 2005), leading to effects on somatic growth (Berge *et al.*, 2006; Thomsen & Melzner, 2010), respiration (Melatunan *et al.*, 2011; Schalkhausser *et al.*, 2013), excretion (Liu & He, 2012), calcification (Gazeau *et al.*, 2007; Wood *et al.*, 2008; Watson *et al.*, 2012) or feeding rates (Bamber, 1990; Navarro *et al.*, 2013). Many marine invertebrates exposed to elevated *p*CO2 have exhibited metabolic depression (Willson & Burnett, 2000; Michaelidis *et al.*, 2005; Navarro *et al.*, 2013) as a decrease in respiration rate while others have remained unaffected (Gutowska *et al.*, 2008; Lannig *et al*., 2010; Clark *et al*., 2013) or even increased their metabolic rate (Wood *et al*., 2008; Beniash *et al*., 2010). These responses are highly species-specific and may vary with organism size (Beniash *et al*., 2010). The resilience of the species studied, and the capacity to regulate metabolism under stressful conditions are also important (Pörtner, 2008). These physiological impacts are likely to have broad effects on the survival, growth and reproduction of marine species (Shirayama & Thornton, 2005; Byrne, 2011), which would lead to changes in community structure from altered diversity and abundances (Hale *et al*., 2011; Kroeker *et al*., 2013a).

These physiological impacts are likely modulated by temperature because temperature is a primary driver of physiological function in ectotherms (Hofmann & Todgham, 2010). Increasing temperature affects the rate of all biochemical reactions, and hence cellular processes and physiological functions (Clarke, 1983; Pörtner, 2012), increasing metabolic costs within a limited thermal tolerance window (Peck *et al*., 2002; Marshall *et al*., 2003). The interactive effects of increased temperature and elevated CO2 concentrations are predicted to impair physiological processes (Clarke, 2003; Pörtner, 2008) by narrowing the thermal tolerance window of the organisms (Metzger et al., 2007; Lannig et al., 2010) and elevating vulnerability to extreme temperature (Schalkhausser et al., 2012).

In a context of global change, non-indigenous species are expected to be favored in their introduced area (Dukes & Mooney, 1999; Occhipinti-Ambrogi, 2007) mainly because robustness to abiotic variation is often a trait that determines the success of invasive of a species (Hellmann *et al*., 2008; Lenz *et al*., 2011). Climatic changes in the physical environment will likely affect the distribution, spread, abundance, impacts and interactions of species, possibly to the advantage of introduced organisms (Occhipinti-Ambrogi, 2007). Thus our study focused on the response of an invasive Calyptraeidae gastropod living on western European coasts, but which originates from North East America. The slipper limpet, *Crepidula fornicata* (Linné 1758) was introduced in Europe at the end of the 19th century, mainly with oysters (*Crassostrea gigas*) which were imported for farming (Blanchard, 1995), and has subsequently colonized European coasts from southern Sweden to southern France (Blanchard, 1997). *C. fornicata* has significant impacts on biodiversity and ecosystem functioning where it has established (De Montaudouin *et al*., 1999; Decottignies *et al.*, 2007; Martin *et al*., 2007). It lives in shallow sites, especially in bays and estuaries where very high densities of over one thousand individuals m-2 have been reported (Blanchard, 1995). *C. fornicata* is known to be strongly resistant to environmental variations, particularly temperature and salinity (Blanchard, 1995; Blanchard, 1997; Diederich & Pechenick, 2013). In light of the different ecological and physiological characteristics of *C. fornicata*, it is important to investigate the impact of future *p*CO2 levels, and determine its resistance capacities to high levels of stress to assess the likely future impact of this engineer species in the ecosystems to which it was introduced.

The present study was designed to investigate the metabolic responses of *C. fornica*ta to high *p*CO2 conditions during temperature stress. Short-term experimental approaches using faster temperature elevations than natural changes provide valuable insight into physiological responses of marine invertebrates in term of their ability to resist high levels of stress or their lethal temperature (Sokolova & Pörtner, 2003; Peck *et al*., 2004; Pörtner *et al*., 2006; Richard *et al*., 2012). Following the hypothesis that CO2 stress will increase sensitivity to temperature change, we evaluated changes in oxygen-consumption of *C. fornicata* individuals previously reared under elevated *p*CO2 for 10 weeks during a rapid temperature increase (1°C 12h-1). Respiration rates were measured as a proxy for metabolism on males (small individuals) and females (large individuals), as in this species there is sexual dimorphism in size.

**FMATERIAL & METHODS**

*Biological material*

*Crepidula fornicata* stacks were collected by SCUBA divers on 4 February 2010, in Morlaix Bay (northwest Brittany, France), at the “Barre des Flots” site (3°53.015'W; 48°40.015'N) at a depth of 10 meters and at an *in situ* temperature of 11.6°C (SOMLIT: *Service d’Observation de la Mer et du LITtoral* data). They were transferred directly to aquaria at the Station Biologique de Roscoff where they were held in natural unfiltered seawater at a temperature around 10°C, until they were used in experiments starting on 10 March 2010.

Males and females at the top and the bottom of stacks respectively, were selected, separated and individually labelled. Small males (23.31 ± 0.16 mm length), which were still slightly mobile, were placed individually on 3 cm Petri dishes one month before the beginning of the trials. Dead individual shells at the base of stacks were kept as the substratum under the largest living immobile females (47.53 ± 0.25 mm length). In *C. fornicata*, size cannot be discriminated from sex because this is a protandrous hermaphroditic organism, changing sex with age and size (Coe 1938). All individuals were gently brushed to remove epibionts and biofilm from their shells before proceeding to the metabolic measurements.

Condition indices (CI) were calculated on a pool of 20 specimens in March, before the beginning of the experiment, and on all remaining living and recently dead individuals (male n = 74; female n = 99) at the end of the temperature increase on 29 May 2010. Shell dry weight (DWShell), shell length and tissue dry weight (DWTissue) were determined separately on each individual after drying at 60°C for 48h. Specimens were then ignited in a muffle furnace at 520°C for 6 h, with tissue ash-free dry weight (AFDWTissue) being obtained by difference. CI were calculated as:

CI = (AFDWTissue / DWShell) × 100.

Mortality was checked daily during the experiment. Individuals with no reaction when the foot was stimulated were classed as dead and removed from the tanks.

*Experimental conditions and set-up*

After distributing randomly in each of twelve 10-L aquarium tanks comprising the experimental flow-through system (as described in Noisette *et al*., 2013), 120 males and 120 females (i.e. 10 individuals of each sex per aquarium) were held in different *p*CO2 conditions between 13 March and 29 May 2010. At the beginning of the experiment, pH was gradually decreased (by bubbling CO2) over four days at 0.1 pH units day-1 from 8.1 until the required pH was reached. Specimens were subsequently held for ten weeks in four different *p*CO2 conditions: a current *p*CO2 of 380 µatm (pHT = 8.07), and three elevated *p*CO2 levelsof550 µatm (pHT = 7.94), 750 µatm (pHT = 7.82) and 1000 µatm (pHT = 7.77). The elevated *p*CO2 values corresponded to different scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC) for the end of the century (Solomon *et al*., 2007) and were selected according to the recommendations of Barry *et al.*, (2010). *p*CO2 was adjusted by bubbling CO2-free air (current *p*CO2) or pure CO2 (elevated *p*CO2) in four 100 L header tanks (1 per *p*CO2 condition) supplied with natural unfiltered seawater pumped from the sea, directly at the foot of the Station Biologique de Roscoff. Seawater was continually delivered by gravity from each header tank to three aquaria per *p*CO2 condition at a constant rate of 9 L h-1 (renewal rate: 90% total aquarium volume h-1). *p*CO2 was monitored and controlled by a feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header tanks. pH values of the pH-stat system were adjusted from daily measurements of pH on the total scale (pHT) in the aquaria using a pH meter (HQ40D, Hach Lange, Ltd portable LDOTM, Loveland, Colorado, USA) calibrated using Tris/HCl and 2-aminopyridine/HCl buffers (Dickson et al., 2007). The twelve aquaria were placed in four thermostatic baths where temperature was controlled to ± 0.2 °C using 100 - 150 W submersible heaters.

Before the rapid temperature increase experiment, *C. fornicata* individuals were maintained in the different *p*CO2 treatments for 10 weeks while temperature was raised successively to mimic the natural rate of temperature change from winter to summer. Temperature was maintained at 10°C from the beginning of the trial to 29 March. It was raised to 13°C from 5 to 19 April and to 16°C from 26 April to 18 May 2010. To reach these set levels the temperature was increased by 0.5°C day-1 until the new set temperature was achieved. During the experiment, animals were naturally fed by the phytoplankton provided by unfiltered seawater.

The rapid temperature increase experiment was conducted between the 18 and 29 May 2010. In all four *p*CO2 treatments, temperature was increased from 16 to 36°C at 1°C 12h-1. *C. fornicata* oxygen consumption was measured (see below) both in small and large individuals in the different *p*CO2 treatments during this rapid temperature increase.

*Seawater parameters*

Seawater parameters were monitored throughout the experiment. pHT and temperature were recorded daily in each of the 12 aquaria using a pH meter (HQ40D, Hach Lange, Ltd portable LDOTM, Loveland, Colorado, USA). Total alkalinity was determined every 3 weeks by 0.01N HCl potentiometric titration on an automatic titrator (Titroline alpha, Schott SI Analytics, Mainz, Germany). Seawater carbonate chemistry, *i.e.* exact CO2 partial pressure (*p*CO2) and saturation state of aragonite were calculated in each *p*CO2 conditionusing CO2SYS software(Lewis & Wallace, 1998) using constants from Mehrbach *et al*., (1973) refitted by Dickson & Millero, (1987). Mean values (± standard error, SE) of the parameters in each *p*CO2 treatment are presented in Table 1.

*Oxygen consumption measurements*

During the rapid temperature increase trial (18 - 29 May 2010), oxygen consumption of 6 randomly selected labeled individuals of each sex (2 per aquaria) was measured in each of the *p*CO2 treatments every two days, at 18, 22, 26, 30 and 34°C. Respiration rates were determined using closed incubations in 75 mL (males) or 180 mL (females) acrylic chambers (Engineering & Design Plastics Ltd, Cambridge, UK) filled with water from the same aquarium (see methods in Morley *et al*., 2007). Chambers were placed in their respective aquaria during incubations to keep the temperature constant. Incubations varied between 1 h and 3 h depending on temperature and were halted before oxygen saturation fell below 80% saturation. Control incubations without animals (n = 1 control incubation / aquarium / measurement) were carried out to allow correction for microbial activity in seawater.

Respiration rates were calculated from the differences in measurements of oxygen concentration during trials and controls using a non-invasive fiber-optical system (FIBOX 3, PreSens, Regensburg, Germany) made up of an optical fiber and reactive oxygen spots attached to the inner wall of the chambers. These spots were calibrated with 0% and 100% oxygen buffers made from the manufacturer instructions. 0% O2 buffer was prepared by dissolving 10 g of Na2SO3 in 1 L of seawater and 100% O2 buffer was prepared by bubbling air in 1L of seawater for 20 min to achieve oxygen saturation. Previous experiments had demonstrated that oxygen consumption remained linear during all the incubation periods. Chamber contents were mixed gently by inverting chambers several times before each oxygen measurement. Respiration (R) rates (in µmol O2 g-1 AFDW h-1) were corrected for oxygen consumption in controls and calculated as:

R = – (ΔO2 × V) / (Δt × AFDWTissue)

where ΔO2 (µmol O2 L-1) is the difference between initial and final O2 concentrations during the incubation, V (L) is the chamber volume minus the individual *C. fornicata* volume, Δt (h) is the incubation time and AFDWTissue (g) is the tissue ash free dry weight of the slipper limpet incubated.

Q10 coefficients were calculated by using the standard equation:

Q10 = (RH/ RL) 10 / (Th – Tl)

where TL and TH were the lowest and highest temperature reached and RL and RH the respiration rates in these temperature respectively.

*Statistical analyses*

All statistical analyses were performed using R version 2.15.0 (R Core Team 2013) and STATISTICA software. A logistic regression (general linear model, GLM) was applied to test the differences in mortalities between the different *p*CO2 treatments and between sex with temperature as the linear variable. The effects of *p*CO2, sex and the interaction of these two factors on condition index (CI) at the end of the experiment and on Q10 values were investigated by 2-way analysis of variance (ANOVA). Linear regressions between respiration rates and increasing temperatures were fitted in the four different *p*CO2 treatments for males and females separately. Differences between *p*CO2 treatments were explored using an ANCOVA with *p*CO2 and sex as fixed factors and temperature as co-variable.. Normality was assessed using the Kolmogorov-Smirnov test and Levene’s test was used to ensure that variances were homogenous. All the results are presented as mean ± standard error (SE).

**RESULTS**

Mortality occurred between 34 and 36°C for females and 22 and 36°C for males (Figure 1). There were no significant differences in mortality between the different *p*CO2 treatments (GLM, df = 3, F = 0.680, p = 0.565) or between males and females (GLM, df = 1, F = 0.580, p = 0.449). Moreover, the interaction between factors *p*CO2 and sex of the individuals was not significant (GLM, df = 3; F = 0.21; p = 0.888). At *p*CO2 levels of 380, 550, 750 and 1000 µatm, the mortality was 29, 19, 19, and 24 for females and 28, 6, 8, and 6 for males . At the end of the acute temperature increase nearly twice the number of females had died (91) compared with the males (48) (χ² test, p < 0.05).

The mean condition index before the start of the experiment was 3.00 ± 0.27 (n=10). It varied at the end of the experiment between 1.69 ± 0.13 for males at *p*CO2 of 380 µatm and 2.41 ± 0.27 for females at *p*CO2 of 550 µatm (Table 2). There were no effects of *p*CO2 , sex or the interaction of these two factors on the condition index at the end of the trial (Table 2). However, the condition index from the beginning of the experiment (3.00 ± 0.27) was different from the mean condition index including all *p*CO2 conditions (2.11 ± 0.07) at the end of the trial (t-test, df = 181, t = 3.159, p = 0.002), which means that CI in both males and females decreased significantly from the start to the end of the experiment (Figure 2).

Female respiration rates varied between 0.51 µmol O2 g-1 AFDW h-1 at 18°C and *p*CO2 of 750 µatm and 91.62 µmol O2 g-1 AFDW h-1 at 32°C and *p*CO2 of 380 µatm. Males had higher rates, which ranged between 5.13 µmol O2 g-1 AFDW h-1 at 18°C and *p*CO2 of 380 µatm and 175.51 µmol O2 g-1 AFDW h-1 at 32°C and *p*CO2 of 380 (Figure 3).

Relationships between respiration rate and temperature were linear at each *p*CO2 level (Figure 3). Respiration rose significantly with increasing temperature in all *p*CO2 treatments, for both males and females (Table 3, all p-values < 0.02). There were no significant differences between the slopes of the different regressions among the *p*CO2 treatments or between sexes (analysis of slopes, df = 3, F = 1.1, p = 0.346). The intercepts of the different regressions also did not significantly vary among *p*CO2 (ANCOVA, df = 3, F = 0.350, p = 0.789), but there were difference between males and females (ANCOVA, df = 1, F = 62.63, p < 0.001).

Q10 values ranged from 1.24 to 2.40 for females and from 1.36 to 2.77 for males among the different *p*CO2 treatments (Figure 2). There was no significant *p*CO2 effect on Q10 values for either males or females(Table 2). Across all *p*CO2 treatments, females had significantly lower Q10 values than males with means of 1.61 ± 0.11 and 2.00 ± 0.12 for females and males, respectively (Table 2). The interaction between *p*CO2 and sex, however, was not significant (Table 2).

**DISCUSSION**

Independently of the impact of *p*CO2 we planned to test, one of the major issues of this study was food limitation which was unintentionally imposed on the *C. fornicata* individuals in the experiments. This food limitation was detected because the decrease in condition indices (CI) of both males and females from the beginning to the end of the experiment. Such decreases in CI are usually related to food quantity or quality supplied to organisms (Norkko & Thrush, 2006). Animals were maintained in unfiltered seawater which carried natural phytoplankton at a concentration between 0.2 and 1 µg Chl a L-1 (*SOMLIT* data). The water renewal in the aquarium was maintained constant at a rate of 0.9 L h-1 (i.e. 90% of the total volume of each aquarium changed per hour). Water supply in our experimental system was likely too low to provide sufficient food for the experimental animals, which thus relied on internal energy reserves and so decreased their CI. A similar outcome was reported for mussels by Mackenzie *et al.* (2014).

The use of stored reserves was similar in the different *p*CO2 conditions as CI at the end of the experiment did not differ between the different *p*CO2 treatments, and this was the case for both sexes. Previous studies have shown interspecific variability in the responses of condition indices under high *p*CO2 levels, ranging from a lack of effect (Cummings *et al*., 2011; Clark *et al*., 2013; Sanders *et al*., 2013) to large changes in condition under high *p*CO2 levels (Hiebenthal *et al*., 2013; Range *et al*., 2014). Energy availability is a major component in mitigating the effects of ocean acidification (Pansch *et al.*, 2014). Studies have shown that an abundant food supply might counteract even overcome the negative effects of high *p*CO2 on adult and juvenile bivalves (Melzner *et al.*, 2011; Thomsen *et al.*, 2013). Thus, it is important to consider that in this study *C. fornicata* were under limited food conditions when interpreting their metabolic responses to elevated *p*CO2 conditions during the temperature rise. The data here are representative of conditions where there is temperature stress and food supplies are limited, conditions that can occur in the field.

The limitation of food supply was not markedly more important in any of our reduced pH conditions as there were no differences in mortality rates between the different *p*CO2 treatments in *C. fornicata* males and females. This is a different outcome to that reported for some other mollusk species held in elevated *p*CO2 levels (Shirayama & Thornton, 2005; Beniash *et al*., 2010). However, similarly to our study, Pansh *et al.,* (2014) showed that food availability had no impact on mortalities of the barnacle *Amphibalanus improvises* held in different *p*CO2 conditions. In the present study, important mortalities started to occur from 32°C and they became larger at and above 34°C for both males and females. These values are consistent with the upper lethal temperature recorded for *C. fornicata* by Diederich & Pechenick, (2013) in a laboratory study investigating a population from Rhode Island, USA, in which only 40% of the adults survived after a 3 h exposure to 34°C, and all died after a 3 h exposure to 36°C. Mortality was higher in females (larger individuals) than in males (small individuals) even if, male started to die at lower temperatures than females. Similarly, Peck *et al*., (2009) demonstrated for 14 species that smaller species survived to higher temperatures than large ones when temperature was raised at 1°C day-1, and Peck *et al.,* (2013) showed that juveniles had higher upper temperature limits than adults in 4 species of marine invertebrates at warming rates of 1°C day-1 and 1°C 3days-1. The mechanisms setting temperature limits at acute rates of warming may not be energy availability (Peck *et al*., 2014) and females, which had more energetic reserves than males, may thus have not had an advantage.

Despite the decreases in CI, mean respiration rates of *C. fornicata* at 18°C and *p*CO2 of 380 µatm were 31 and 26 µmol O2 g-1 AFDW h-1 for males and females, respectively, which are close to the middle of the range of *in situ* values reported for wild individuals from the Bay of Brest (Brittany, France) (6 to 63 µmol O2 g-1 AFDW h-1: Martin *et al*., 2006). This indicates that animals in the experiments here had similar oxygen consumption than wild specimens and were not metabolically depressed under insufficient food supply. In both *C. fornicata* males and females, respiration rates increased with temperature, as previously demonstrated for this species by Newell & Kofoed, (1977) and most ectotherm metabolic rates are correlated positively with temperature (Cossins & Bowler, 1987). Respiration rates were higher in *C. fornicata* males than in females regardless of the temperature. Generally, mass-specific respiration rates of small individuals are higher than those of larger ones because metabolic rate (normalized to the biomass) decreases with increasing organisms size (von Bertalanffy, 1951; Parsons *et al*., 1984).

The relationship between oxygen consumption and temperature here for *C. fornicata* was similar in all the different *p*CO2 treatments. The slopes and intercepts of the regressions were not significantly different across the four *p*CO2 conditions which means temperature effect on respiration rate was not affected by the different *p*CO2 levels in males or females. In constrast to our results, Lannig *et al*., (2010) found that an acute temperature rise (1.25°C/12h) caused a more rapid increase in metabolic rate in *Crassostrea gigas* under elevated *p*CO2 conditions, and there was asynergistic effect of temperature and *p*CO2. The lack of difference in respiration between animals held in different *p*CO2 conditions may be related to a stronger ability to up-regulate their metabolism under a temperature stress irrespective of *p*CO2. Thus, under warming conditions, *C. fornicata* can generate sufficient energy to cope with any effects of decreased pH (Wood *et al*., 2010). *Q*10 values were also similar across *p*CO2 treatments in both males and females and they were within the expected range of values recorded for marine invertebrates (Branch *et al*., 1988; Marshall *et al*., 2003). Even if *C. fornicata* individuals were food limited, their oxygen consumption remained unaffected by elevated *p*CO2. A similar lack of *p*CO2 effect was reported for growth and shell strength of the barnacle *A. improvisus* (Pansch *et al.*, 2014). In our study, the low food supply did not appear to affect the resistance or resilience of *C. fornicata* to CO2 stress.

Several studies investigating the response of mollusk respiration to elevated *p*CO2 have demonstrated metabolic depression under high *p*CO2 in both bivalves and gastropods (Michaelidis *et al*., 2005; Bibby *et al*., 2007; Fernandez-Reiriz *et al*., 2011; Melatunan *et al*., 2011; Liu & He, 2012; Navarro *et al*., 2013). Conversely, others observed no *p*CO2 effect on mollusk respiration and general metabolism (Gazeau *et al*., 2007; Marchant *et al*., 2010; Fernandez-Reiriz *et al*., 2012; Clark *et al*., 2013) as reported in our study. In some rare cases, O2 consumption was reported to increase under high *p*CO2 conditions (Wood *et al*., 2010; Cummings *et al*., 2011). The effects of high CO2 concentrations on metabolism appear species-specific and depend on resistance capacities of the organisms (Melzner *et al*., 2009). It has been widely reported that exposure to environmental high *p*CO2 levels leads to changes in homeostasis and extracellular acid-base balance counterbalanced by metabolic depression in many cases (Pörtner *et al*., 2005; Pörtner, 2008), although it should be noted, as above, that metabolic depression is often not seen in high *p*CO2 conditions. Differences in acid-base regulatory capacities by increasing HCO3- internal concentrations (Michaelidis *et al*., 2005; Gutowska *et al*., 2010) or H+ excretion (Pörtner *et al*., 2005) are taxon specific and are more or less effective in mitigating the effects of hypercapnia. It has also been suggested that organisms could maintain low metabolic rates without controlling internal pH by not using pH-sensitive oxygen-binding pigments (Thomsen *et al*., 2010; Hiebenthal *et al*., 2013). Such mechanisms may be crucial factors in explaining the observed variation in sensitivities and resistances of marine invertebrates to elevated *p*CO2 conditions (Gutowska *et al*., 2010).

It is important to note here that many of the studies to date on the effects of elevated *p*CO2 on organisms are short-term and acute (e.g. Tomanek *et al*., 2011), not reflecting the long-term trade off in energy balance and physiological changes associated with acclimation of new environmental conditions (Clark *et al*., 2013). For example, metabolic depression acts as a time-limited compensation strategy to survive unfavorable condition such as high CO2 concentrations (Guppy & Withers, 1999; Willson & Burnett, 2000). Because *C. fornicata* were held for 10 weeks in the different *p*CO2 treatments in this investigation, it is likely there was enough time for them to acclimate to the new pH, and no difference in oxygen consumption was detected between the different *p*CO2 conditions. However, the energetic cost likely produced by the negative effects of elevated *p*CO2 may either be relatively small , or difficult to maintain over longer time periods. This could be seen in impacts on other physiological processes than respiration (Catarino *et* *al*., 2012). For example, Bibby *et al*., (2008) demonstrated that exposure to hypercapnic conditions may compromise the ability to express an immune response in mussels. They showed that *Mytilus edulis* phagocytosis declined as function of decreased pH. In the same way, Matozzo *et al*., (2012) showed that elevated *p*CO2 and temperature may strongly affect haemocyte functionality in the bivalves *Chamelea gallina* and *Mytilus galloprovincialis*. Other cellular processes have also been shown to be negatively impacted by high CO2 concentrations, including protein synthesis in the sipunculid *Sipunculus nudus* (Langenbuch *et al*., 2006) or enzyme activities in *C. gallina* and *M. galloprovincialis* (Matozzo *et al*., 2013). However, studies of the impact of reduced pH on immune systems have generally been of short duration and it would be interesting to investigate other physiological parameters than respiration (e.g. calcification, protein production, immunity regulation, fertility) in *C. fornicata* acclimated over several months in the different *p*CO2 conditions predicted for the end of the century. As a coastal species adapted to relatively large fluctuations of abiotic parameters, *C. fornicata* in this study were strongly resistant to both elevated *p*CO2 and increased temperature. Indeed, resistance to high *p*CO2 levels can also come from pre-acclimation or pre-adaptation to fluctuations in the environment where species live (Burnett, 1997). Species living in environments with large abiotic variation have a high phenotypic plasticity which can allow them to survive in stressful conditions (Hofmann & Todgham, 2010). Coastal organisms are more exposed to physico-chemical variations than their open-ocean counterparts that live in more stable thermal and pH environments (Berge *et al*., 2006; Peck *et al*., 2006). Species living in shallow waters tolerate not only seasonal and extreme temperature events but also periodic large fluctuations in seawater pH, driven by biological process that sequester and release large amounts of CO2 (Beniash *et al*., 2010). This exposure to a wide environmental variation has likely led to the evolution of resistance mechanisms to abiotic factors including variations in *p*CO2 and/or pH (Lannig *et al*., 2010).

*C. fornicata* is an invasive species which has successfully colonized European coastal shallow waters. This species is likely to have high phenotypic plasticity and resilience to physico-chemical variations that determined its success. Indeed, successful invasive species generally share characteristics that allow them to establish, colonize and expand their range. Among these characteristics, tolerance to environmental stress is one of the most common (Lenz *et al*., 2011). In a global change context, the movement of physico-chemical conditions away from the optimum increases the energy required by marine species to fuel the extra processes entrained to resist the stresses involved and to maintain homesostasis. This may result in changes in overall physiological condition (Cummings *et al*., 2011) that could impact ecological processes and community interactions. The high resilience to altered *p*CO2/low pH levels observed here for *C. fornicata* may confer a competitive advantage to this invasive species over taxonomically or functionally related species (Lenz *et al*., 2011). For example, the performance of the scallop *Pecten maximus,* which is one of the *C. fornicata* competitors (Thouzeau *et al*., 2000; Fresard & Boncoeur, 2006), has been shown to be negatively affected by high *p*CO2 levels (Schalkhausser *et al*., 2013). These different sensitivities to environmental factors will likely dictate “winners” and “losers” among marine species that could lead to a restructuring of benthic communities. With other studies, our data suggest this restructuring could favor invasive species as evidence is building that shows they are more resistant to change than their native competitors (Dukes & Mooney, 1999; Occhipinti-Ambrogi, 2007).

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**FIGURES CAPTIONS**

**Figure 1:** Cumulated mortalities during the temperature increase. Males are represented on the graph on the top and females are on the graph in the bottom. The greyscale represent the different *p*CO2 levels in which *C. fornicata* individuals where held during the experiment.

**Figure 2:** Mean (± SE) conditions indices at the beginning (black bar), and at the end of the experiment for *C. fornicata* females (white bars) and males (grey bars) in the different *p*CO2. 27 > N > 10

Figure 3: Respiration rates as a function of increasing temperature in each *p*CO2 treatment, for *C. fornicata* males (top, triangles) and females (bottom, circles). Detailed statistical analyses relative to the regressions can be found in Table 3.

**Figure 4:** Mean (± SE) Q10 values for *C. fornicata* females (white bars) and males (grey bars) in the different *p*CO2 treatments. N = 3

TABLES

Table 1: Mean (± standard error, SE) carbonate chemistry parameters for each *p*CO2 treatment. pH (on the total scale, pHT) was measured daily and total alkalinity (AT) was measured every 3 weeks. Other parameters were calculated with CO2sys software. *p*CO2 : CO2 partial pressure; ΩAr : saturation state of seawater with respect to aragonite.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| *p*CO2 treatment |  | pHT |  | *p*CO2 (µatm) |  | ΩAr |  | AT (µEq kg-1 SW) |
|  |  | n = 69 |  | n = 69 |  | n = 69 |  | n = 76 |
| 380 µatm |  | 8.13 ± *0.01* |  | 324 ± *8* |  | 2.72 ± *0.06* |  | 2333 ± *1* |
| 550 µatm |  | 7.89 ± *0.01* |  | 619 ± *16* |  | 1.69 ± *0.04* |  | 2334 ± *2* |
| 750 µatm |  | 7.75 ± *0.01* |  | 873 ± *20* |  | 1.28 ± *0.03* |  | 2335 ± *2* |
| 1000 µatm |  | 7.66 ± *0.01* |  | 1138 ± *65* |  | 1.05 ± *0.02* |  | 2334 ± *2* |

Table 2: Summary of two-way ANOVAs testing the effects of *p*CO2, sex and the interaction of these two factors on the final condition indices (CI) and the Q10 values determined for *C. fornicata* males and females in the different *p*CO2 conditions (380, 550, 750 and 1000 µatm). Bold numbers indicate significant level greater than 95%.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  | **CI** | |  | **Q10** | |  |
|  |  | df |  | F-value | p-value |  | F-value | p-value |  |
|  |  |  |  |  |  |  |  |  |  |
|  | *p*CO2 | 3 |  | 1.245 | 0.295 |  | 0.657 | 0.590 |  |
|  | sex | 1 |  | 2.472 | 0.118 |  | 6.124 | **0.025** |  |
|  | *p*CO2 x sex | 3 |  | 1.371 | 0.254 |  | 2.293 | 0.117 |  |
|  |  |  |  |  |  |  |  |  |  |

Table 3: Relationships between *C. fornicata* male and female respiration rates and temperature in each *p*CO2 treatment

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | *p*CO2 | Regression equation | n | R | R² | F | p |  |
|  |  |  |  |  |  |  |  |  |
| males | 380 | y = 3.691 x - 34.455 | 42 | 0.60 | 0.37 | 22.97 | < 0.001 |  |
| 550 | y = 2.993 x - 18.461 | 42 | 0.46 | 0.21 | 10.56 | 0.002 |  |
| 750 | y = 2.406 x - 4.543 | 41 | 0.40 | 0.16 | 7.55 | 0.009 |  |
| 1000 | y= 3.701 x - 41.556 | 41 | 0.56 | 0.31 | 17.37 | < 0.001 |  |
|  |  |  |  |  |  |  |  |  |
| females | 380 | y = 1.826 x - 7.635 | 42 | 0.49 | 0.24 | 12.72 | < 0.001 |  |
| 550 | y = 1.585 x - 4.218 | 42 | 0.55 | 0.30 | 16.89 | < 0.001 |  |
| 750 | y = 2.637 x - 26.240 | 42 | 0.63 | 0.40 | 26.66 | < 0.001 |  |
| 1000 | y = 1.442 x + 3.435 | 42 | 0.37 | 0.14 | 6.26 | 0.017 |  |
|  |  |  |  |  |  |  |  |  |

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