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To cite this version:
Fanny Noisette, François Bordeyne, Dominique Davoult, Sophie Martin. Assessing the physiological responses of the gastropod Crepidula fornicata to predicted ocean acidification and warming. Limnology and Oceanography, Association for the Sciences of Limnology and Oceanography, 2015, 61 (2), pp.430-444 10.1002/lno.10225. hal-01233060

HAL Id: hal-01233060
https://hal.sorbonne-universite.fr/hal-01233060
Submitted on 24 Nov 2015

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Assessing the physiological responses of the gastropod *Crepidula fornicata* to predicted ocean acidification and warming

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Running title: Responses of *C. fornicata* to OA and warming

Type of paper: Primary Research Article
Organisms inhabiting coastal waters naturally experience diel and seasonal physico-chemical variations. According to various assumptions, coastal species are either considered to be highly tolerant to environmental changes or, conversely, living at the thresholds of their physiological performance. Therefore, these species are either more resistant or more sensitive, respectively, to ocean acidification and warming. Here, we focused on Crepidula fornicata, an invasive gastropod that colonized bays and estuaries on northwestern European coasts during the 20th century. Small (< 3 cm in length) and large (> 4.5 cm in length), sexually mature individuals of C. fornicata were raised for 6 months in three different pCO2 conditions (390, 750 and 1400 µatm) at four successive temperature levels (10, 13, 16 and 19°C). At each temperature level and in each pCO2 condition, we assessed the physiological rates of respiration, ammonia excretion, filtration and calcification on small and large individuals. Results show that, in general, temperature positively influenced respiration, excretion and filtration rates in both small and large individuals. Conversely, increasing pCO2 negatively affected calcification rates, leading to net dissolution in the most drastic pCO2 condition (1400 µatm) but did not affect the other physiological rates. Overall, our results indicate that C. fornicata can tolerate ocean acidification, particularly in the intermediate pCO2 scenario. Moreover, in this eurythermal species, moderate warming may play a buffering role in the future responses of organisms to ocean acidification.

Keywords: calcification, coastal system, invasive species, metabolism, mollusk, pCO2, temperature
Introduction

Predictions indicate that coastal ecosystems will be strongly affected by ocean acidification and warming, currently two of the most prominent anthropogenic processes influencing marine life (Harley et al. 2006). Due to the increase in atmospheric CO$_2$ partial pressure ($p$CO$_2$), pH in surface waters is predicted to decline by 0.06 to 0.32 units and sea surface temperatures to increase by 1.0 to 3.7°C by the end of the century, depending on the Intergovernmental Panel on Climate Change (IPCC) representative concentration pathway considered (Stocker et al. 2013). Modifications in seawater carbonate chemistry due to ocean acidification lead to a decrease in carbonate ion concentrations (CO$_3^{2-}$) (Orr et al. 2005) and a reduction in the calcium carbonate saturation state ($\Omega$), which regulates the thermodynamics of calcium carbonate (CaCO$_3$) precipitation (Feely et al. 2009). In estuarine and coastal waters, pH is more variable than in the open ocean due to intense biological and biogeochemical processes (Andersson and Mackenzie 2011). In these habitats, ocean acidification and warming will shift the baselines, exacerbate natural variations in pH and temperature, and probably threaten the communities living there (Waldbusser and Salisbury 2013).

Mollusks constitute a major taxonomic group in estuarine and coastal waters in terms of community structure and ecosystem functioning (Gutiérrez et al. 2003). Because most marine mollusk taxa accumulate significant amounts of CaCO$_3$ to form protective external shells, they may be sensitive to the changes in pH and carbonate chemistry induced by ocean acidification (for review, see Gazeau et al. 2013), although recent studies have shown that some species could be resistant to elevated $p$CO$_2$ (Range et al. 2011; Ries et al. 2009). Along with direct impacts on calcification, high CO$_2$ concentrations may also have indirect effects on metabolism by disturbing the extracellular acid-base equilibrium, leading to general
internal acidosis (Melzner et al. 2009). These potential shifts in acid-base homeostasis have the potential to change organisms’ energy balance (Pörtner et al. 2005).

In mollusks, the effects of elevated $pCO_2$ and/or decreased pH alone are highly species-specific (see review in Gazeau et al. 2013), and depend on species sensitivity and any existing compensation mechanisms (Michaelidis et al. 2005). To better estimate future ocean acidification effects on mollusk species, various physiological processes have been studied in bivalves and gastropods such as respiration (Beniahs et al. 2010; Bibby et al. 2007), excretion (Fernandez-Reiriz et al. 2011; Liu and He 2012), feeding (Fernandez-Reiriz et al. 2012; Marchant et al. 2010), immune response (Bibby et al. 2008; Matozzo et al. 2012) and protein or enzyme production (Matozzo et al. 2013; Tomanek et al. 2011). However, few studies have simultaneously assessed the responses of more than three physiological processes to ocean acidification and warming. The concomitant increase in seawater temperature and $pCO_2$ are likely to affect mollusk metabolism because, in addition to changes in gas solubility and the proportion of carbon species (Zeebe 2011), temperature also strongly affects physiological and biochemical reactions (Cossins and Bowler 1987). Because warming can modulate the metabolism responses to ocean acidification (Ivanina et al. 2013; Melatunan et al. 2013), investigations of both pH and temperature effects are valuable for understanding the responses of mollusks in the future ocean.

One of the most abundant and widespread shelled mollusks on the French northwestern Atlantic and Channel coasts is the slipper limpet *Crepidula fornicata*, Linnaeus 1758 (Blanchard 1997). This gastropod native to the northeastern American coast was introduced in Europe at the end of the 19th century, mainly via oysters imported for farming (Blanchard, 1995). It then colonized European coasts from southern Sweden to southern France, becoming invasive in some places (Blanchard 1997). *C. fornicata* lives in shallow sites, especially in bays and estuaries where it can reach very high densities of more than 1000
individuals per m$^2$ (Blanchard 1995). This species is known to be highly robust to environmental stress, in particular temperature and salinity (Diederich and Pechenik 2013; Noisette et al. 2015), parameters that have diel and seasonal variations in these coastal habitats. Established *C. fornicata* populations have largely affected biodiversity and ecosystem functioning in terms of sediment modifications (Ehrhold et al. 1998), changes in faunal assemblages (De Montaudouin et al. 1999) and trophic structure (Chauvaud et al. 2000). This species also affects benthic biogeochemical cycles by enhancing filtration, metabolic activities, CaCO$_3$ production, and the recycling of nutrients and dissolved carbon back into the pelagic ecosystem (Martin et al. 2006; Martin et al. 2007; Ragueneau et al. 2002).

Although *C. fornicata* is likely highly tolerant to environmental fluctuations, the combined effects of decreased pH and increased temperature may push this species away from its physiological optimum. Thus the objective of this work was to quantify the respiration, ammonia excretion, filtration and calcification responses of small and large specimens of *C. fornicata* in different temperature and $p$CO$_2$ conditions. Investigating the physiology of this key engineer in some coastal ecosystems in a context of climate change is one way to better understand the sensitivity of this species and its potential future ecological impact.

**Methods**

**Sampling site and in situ conditions**  
*C. fornicata* stacks were collected by SCUBA divers on 30 November 2011, in Morlaix Bay (northwestern Brittany, France), at the “Barre des Flots” site (3°53.015'W; 48°40.015'N) at approximately 11 m depth. No temporal series of abiotic parameters were
available for this exact location. However, variations in the physico-chemical parameters (surface measurements) at a station (called Estacade), located approximately 10 km from the Barre des Flots site, were obtained from the Service d’Observation des Milieux LITorraux (SOMLIT) between 2010 and 2013, with a sampling step of 15 days. Between October 2010 and March 2013, temperature varied between 8.1°C (January 2011) and 16.5°C (August 2011) with mean values (± SE) of 10.1 ± 0.2°C in winter, 12.7 ± 0.4°C in spring and 15.8 ± 0.02°C in summer.

In Morlaix Bay (2009 to 2011), phytoplankton groups (> 5µm), the most important food resource of *C. fornicata* (Decottignies et al. 2007), were mainly dominated by planktonic diatoms in concentrations varying between 10 to 300 cells mL\(^{-1}\) (depending on the season) and dinoflagellate species that were found at lower abundances (ca. 25 cells mL\(^{-1}\); Leroy 2011).

**Biological material**

*C. fornicata* forms stacks of several individuals in which each individual adheres to the dorsal surface of the shell of the subjacent partner in the stack. It is a protandrous hermaphrodite, meaning that the small individuals at the top of the stacks are generally males and the large ones at the bottom, females (Coe 1936). After sampling, stacks were brought directly to the *Station Biologique de Roscoff* where they were kept in natural, unfiltered seawater for 6 weeks at a temperature gradually lowered to 10°C, reflecting the seasonal drop in temperature between autumn and winter. Sexually mature individuals (more than 1 cm in length) were selected and separated into two class sizes: small individuals (29.5 ± 0.9 mm length) from the top of the stack and larger ones (45.4 ± 0.6 mm length) from the bottom. They were separated from the stack and individually labeled with tags glued on their shell. Empty subjacent shells, whose soft tissue was removed, served as substratum for the sampled
live individuals. Other empty shells whose size was similar to that of the substratum shell of live individuals were also selected for flux corrections (see part “Metabolic rates and O:N ratios” below). All the shells were gently brushed to remove epibionts without altering periostracum layer.

Length (in mm), volume (in mL) and tissue dry weight (DW in g) of the live individuals were determined for each incubated specimen at the end of the whole experiment. Length was measured with calipers, volume was estimated as the volume of seawater moved when individual was immersed and DW was determined after drying fresh samples at 60°C for 48 h.

Experimental conditions

Single small and large individuals, along with their substratum shell, were randomly distributed into nine 10 L aquaria with 10 individuals of each class size per each aquarium. Empty shells were also distributed into nine other 10 L aquaria (4 shells per aquarium). At the beginning of the experiment, pH was gradually decreased over 2 weeks by 0.02 pH unit per day from 8.1 until the different pH treatments were reached. *C. fornicata* individuals and empty shells were then subsequently held for 24 weeks (12 January to 27 June 2012) in three \( pCO_2 \) treatments selected according to the recommendations in Barry et al. (2010): (1) 390 \( \mu \text{atm (pH}_T = 8.07 \) represented current \( pCO_2 \), (2) 750 \( \mu \text{atm (pH}_T = 7.82 \) corresponded to the elevated \( pCO_2 \) level predicted by the IPCC for the end of the century (Solomon et al. 2007) and (3) 1400 \( \mu \text{atm (pH}_T = 7.56 \) represented a \( pCO_2 \) five-fold higher than preindustrial \( pCO_2 \) (280 \( \mu \text{atm) also predicted for 2100 (Stocker et al. 2013). pCO}_2 \) was adjusted by bubbling \( CO_2 \)-free air (current \( pCO_2 \) or pure \( CO_2 \) (elevated \( pCO_2 \)) in three 100 L header tanks supplied with unfiltered seawater pumped directly from the foot of the *Station Biologique de Roscoff*.

Each of the three \( pCO_2 \) treatments had six replicate 10 L aquaria, three for live organisms and
three for empty shells. They continuously received CO$_2$-treated seawater at a rate of 9 L h$^{-1}$ (i.e. a renewal rate of 90% h$^{-1}$) from the header tanks. $p$CO$_2$ was monitored and controlled by an offline feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header tanks. The pH values of the IKS system were adjusted from daily measurements of $p$H$_T$ in the 18 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris HCl and 2-aminopyridine HCl buffers (Dickson et al. 2007).

In each $p$CO$_2$ treatment, temperature was raised from 10 to 19°C with an incremental step of 3°C. The first three temperature levels (10 to 16°C) simulated the natural change in temperature from winter to summer in Morlaix Bay whereas the last level (19°C) corresponded to a temperature increase of 3°C predicted for the end of the century (Solomon et al. 2007). *C. fornicata* individuals were held for three weeks at each temperature before carrying out the metabolic measurements (see below). This acclimation time was long enough to overcome the immediate stress response (Meistertzheim et al. 2007). Temperature was maintained at (1) 10°C (1$^{\text{st}}$ trial period) from 16 January to 12 February 2012; (2) 13°C (2$^{\text{nd}}$ trial period) from 27 February to 25 March 2012; (3) 16°C (3$^{\text{rd}}$ trial period) from 9 April to 6 May 2012, and (4) 19°C (4$^{\text{th}}$ trial period) from 21 May to 27 June 2012. Between two temperature levels, temperature was gradually increased by 0.2°C day$^{-1}$ over two weeks. The 18 aquaria were placed in thermostatic baths in which temperature was regulated to within ± 0.2°C using submersible 150 to 250 W heaters controlled by the IKS system.

Three independent 10 L aquaria named “control” were maintained at 10°C under ambient pH (with no $p$CO$_2$ control) until the end of the experiment in order to estimate a potential bias on metabolism induced by the mesocosm experiment over time. Each aquarium contained 10 small and 10 large slipper limpets on their substratum shell and was supplied
with the same seawater sourced from the header tanks. They were kept in a thermostatic bath
regulated at 10°C by an aquarium chiller (TC5, TECO®, Ravenna, Italy).

In addition to the natural phytoplankton found in the unfiltered seawater, all slipper
limpets were fed twice a week with a stock solution composed of the diatom *Chaetoceros
gracilis* (~ $15 \times 10^6$ cells mL$^{-1}$) and the dinoflagellate *Isochrysis affinis galbana* (~ $26 \times 10^6$
cells mL$^{-1}$); 400 mL of this microalgal mix was added to each aquarium at each feeding.

Seawater flow was stopped for 2 h when organisms were fed and filtering actively. During
this feeding time, pH variation did not exceed 0.05 units.

Individuals that did not adhere to their substratum shell and that showed no reaction
when their foot was stimulated were counted as dead and removed from the tanks. Mortality
reached only 8% at the end of the experiment among all pCO$_2$ conditions.

**Seawater parameter monitoring**

Seawater parameters were monitored throughout the experiment. pH$_T$ and temperature
were recorded daily in each of the 21 aquaria (18 + 3 controls) using a pH meter (826 pH
mobile, Metrohm AG, Herisau, Switzerland) as described above. Total alkalinity (A$_T$) was
measured at each trial period by 0.01 N HCl potentiometric titration on an automatic titrator
(Titroline alpha, Schott SI Analytics, Mainz, Germany). Salinity was also measured at each
trial period with a conductimeter (LF 330/ SET, WTW, Weilheim, Germany). Seawater
carbonate chemistry, i.e. dissolved inorganic carbon (DIC), pCO$_2$ and the saturation state of
aragonite ($\Omega_{ar}$) were calculated for each pCO$_2$ level and temperature with CO$_2$SYS software
(Lewis and Wallace 1998) using constants from Mehrbach et al. (1973) refitted by Dickson &

**Metabolic rates and O:N ratios**
Metabolic rates were assessed at each temperature level after a four-day starvation period and after the shells were gently cleaned to remove biofilm-forming organisms. Two small and two large individuals were selected per aquarium. They were incubated individually in 185 mL (small) and 316 mL (large) acrylic chambers (Engineering & Design Plastics Ltd, Cambridge, UK) filled with seawater from their respective aquaria. They were put on a plastic grid above a stirring bar (speed 100 rpm.), which ensured water homogeneity. Chambers were placed in their original aquaria for incubation to keep the temperature constant. Incubations were carried out in dark for 2 to 10 h, depending on temperature and limpet size, to maintain oxygen saturation above 80% until the end of the incubation. At each temperature period, empty shell incubations were carried out to correct individual rates for fluxes related to the substratum shell. Blank incubations containing only seawater from the aquarium also helped to correct fluxes for any microbiological activity in seawater.

Oxygen concentrations were measured at the beginning and the end of the incubation period with a non-invasive fiber-optics system and reactive oxygen spots attached to the inner wall of the chambers (FIBOX 3, PreSens, Regensburg, Germany). Spots were calibrated at the beginning of each trial period with 0% and 100% oxygen buffers. Seawater was sampled for ammonium (NH$_4^+$) concentration and A$_T$ measurements with 100 mL syringes at the beginning of the incubation, directly in the aquaria just after the chambers were closed, and at the end of the incubation, in the incubation chamber itself. Samples were filtered through 0.7 μm Whatman GF/F filters into 100 mL glass bottles and fixed with reagent solutions for ammonium or poisoned with mercuric chloride (0.02% vol/vol; Dickson et al. 2007) for A$_T$ measurements. Vials were stored in the dark pending analysis. NH$_4^+$ concentrations were then determined using the Solorzano method (Solorzano 1969) based on spectrophotometry at a wavelength of 630 nm (spectrophotometer UV-1201V, Shimadzu Corp, Kyoto, Japan). A$_T$ (in μEq L$^{-1}$) values were determined by 0.01 N HCl potentiometric titration on an automatic
titrator (Titroline alpha, Schott SI Analytics, Mainz, Germany) and by using the Gran method (non-linear least-squares fit) applied to pH values from 3.5 to 3.0 (Dickson et al. 2007).

Respiration (in µmol O₂ g⁻¹ DW h⁻¹; equation [1]) and excretion (in µmol NH₄⁺ g⁻¹ DW h⁻¹; equation [2]) were directly calculated from oxygen and ammonium concentrations, respectively. Net calcification (in µmol CaCO₃ g⁻¹ DW h⁻¹; equation [3]) was estimated using the alkalinity anomaly technique (Smith and Key 1975) based on a decrease in A_T by 2 equivalents for each mole of CaCO₃ precipitated (Wolf-Gladrow et al. 2007). As ammonium production increases alkalinity in a mole-per-mole ratio (Wolf-Gladrow et al. 2007), the alkalinity variation was corrected by the ammonium flux to calculate CaCO₃ fluxes.

\[ [1] \quad R = \frac{\Delta O_2 \times V}{\Delta t \times DW} \]

\[ [2] \quad E = \frac{\Delta NH_4^+ \times V}{\Delta t \times DW} \]

\[ [3] \quad G_n = -\frac{(\Delta A_T - \Delta NH_4^+) \times V}{2 \times \Delta t \times DW} \]

where \( \Delta O_2 \) (in µmol O₂ L⁻¹) is the difference between initial and final O₂ concentrations; \( \Delta NH_4^+ \) (in µmol NH₄⁺ L⁻¹) is the difference between initial and final NH₄⁺ concentrations; \( \Delta A_T \) is the difference between initial and final total alkalinity (µmol Eq L⁻¹); V (in L) is the volume of the chamber minus C. fornicata volume; \( \Delta t \) (in h) is the incubation time and DW (in g) is the soft tissue dry weight of incubated C. fornicata.

In addition, oxygen consumption of the individuals maintained at 10°C during the experiment were assessed on six small and six large individuals at each trial period, following the technique described above. These “controls” tested if mesocosm conditioning induced metabolic stress over time.

The O:N ratio, which corresponds to the amount of oxygen consumed for nitrogen excreted, was calculated from respiration and excretion rates except for the experiments run at 10°C for which rates were too low to obtain significant data. Generally, the O:N ratio is
considered a common indicator of the proportion of the three metabolic substrates (carbohydrates, lipids and proteins) used in energy metabolism (Mayzaud and Conover 1988). The atomic ratio of oxygen uptake and excreted nitrogen was calculated following the equation [4] based on Thomsen & Melzner (2010):

[4]: $\text{O:N} = \frac{R}{E}$

where R is the respiration rate used as a proxy of the quantity of oxygen consumed by the individual and E, the excretion rate representing the concentration of nitrogen excreted.

Filtration rates

At each trial period, the filtration rate of three small and three large slipper limpets per $p\text{CO}_2$ condition (i.e. 1 individual per size per aquarium) was determined by calculating clearance rates (Coughlan 1969). To do so, 10 and 20 mL of a microalgae mix ($C.\text{gracilis, T. affinis galbana}$, 1:1) were added to the small and large chambers (same as for metabolic measurements), respectively, using a 10 mL syringe equipped with a thin tube. The mean initial concentration of the mix was $1\ 200\ 000 \pm 310\ 000$ cell mL$^{-1}$. In parallel, control incubations containing only microalgae were carried out to check that phytoplankton cells did not multiply significantly during the incubation. Water from the chambers was sampled with the syringe every 15 min until the water became totally clear (around 2 h). Samples were immediately fixed with 25% glutaraldehyde and frozen at $-80^\circ\text{C}$ pending analyses (Marie et al. 1999). The number of microalgal cells in each sample was then determined on 200 µL aliquots using flux cytometry (Cell Lab Quanta™, SC, Beckman Coulter, USA). Filtration rates (F, in mL SW g$^{-1}$ DW min$^{-1}$) were calculated following equation [5]:

[5] $F = V \times \frac{\ln[C_i] - \ln[C_f]}{\Delta t \times DW}$

where [Ci] and [Cf] (in cell mL$^{-1}$) were respectively the initial and final cell concentrations in the chamber water; V (in L) is the volume of the chamber minus individual $C.\text{fornicata}$
volume; Δt (in h) is the incubation time and DW (in g) is the tissue dry weight of the individual incubated.

Statistical analyses

All statistical analyses were performed using the R software, version 2.15.0 (R Core Team 2013). Normality and homoscedasticity were checked using Kolmogorov-Smirnov’s test and Levene’s test, respectively, before each statistical test. Spatial pseudoreplication effect was first tested by considering “aquarium” as a random factor (p-value < 0.05). Then, statistical analyses were simplified to two-way ANOVAs with repeated measurements on the same individual through the four trial periods (different temperature levels) separately for small and large individuals. These analyses were performed for the four physiological rates (respiration, excretion, calcification and filtration) and the O:N ratio, assuming pCO$_2$ and temperature as fixed factors. Student-Newman-Keuls (SNK) post hoc tests were applied to identify differences among treatments with a confidence level of 95% when ANOVA showed significant results. In parallel, any changes in the respiration rate of individuals constantly maintained at 10°C through time were assessed using a non-parametric Friedman test for repeated measurements, separately for small and large slipper limpets. All results are given as mean ± standard error (SE).

Results

Seawater parameters

The mean temperature and carbonate chemistry parameters among the pCO$_2$ and temperature conditions are presented in Table 1. Temperature was stable at each trial period with a variability lower than 0.5°C. The different pCO$_2$ levels remained close to the selected
values of 390, 750 and 1400 µatm except at 19°C where all pCO₂ increased from the baseline (+ 100-200 µatm). A_T ranged from 2365 ± 2 to 2422 ± 2 µEq kg⁻¹. Ω_A decreased by less than 1 only in the 1400 µatm pCO₂ condition. Salinity varied between 34.2 ± 0.1 and 35.1 ± 0.1 among the different pCO₂ and temperature levels with no effect of the temperature increase on salinity.

Respiration, excretion and O:N ratio

Respiration and excretion rates changed significantly with temperature, but not with pCO₂, in small and large individuals (Figure 1, Table 2). After pooling results for all pCO₂ conditions, mean respiration rates in small C. fornicata increased from 3.78 µmol O₂ g⁻¹ DW h⁻¹ at 10°C to 11.76 µmol O₂ g⁻¹ DW h⁻¹ at 19°C. In large individuals, the lowest mean respiration rate was recorded at 10°C (4.82 µmol O₂ g⁻¹ DW h⁻¹) whereas rates did not differ from 13 to 19°C with a mean value of 11.50 µmol O₂ g⁻¹ DW h⁻¹. Oxygen fluxes measured on empty shells represented only 4% of the whole organism fluxes measured and decreased only slightly with temperature.

Mean excretion rates calculated among pCO₂ conditions for small C. fornicata individuals gradually increased from 0.15 µmol NH₃ g⁻¹ DW h⁻¹ at 10°C to 1.47 µmol NH₃ g⁻¹ DW h⁻¹ at 19°C. Excretion rates of large individuals showed a parabolic trend with an increase from 10°C (0.16 µmol NH₃ g⁻¹ DW h⁻¹) to 16°C (1.34 µmol NH₃ g⁻¹ DW h⁻¹) followed by a decrease at 19°C (0.74 µmol NH₃ g⁻¹ DW h⁻¹). The ammonium fluxes of empty shells represented less than 1% of the fluxes estimated for whole organisms and were higher at 10°C than at the other temperature levels (rates practically nil).

O:N ratios varied greatly, ranging from 2.86 to 31.68 with a mean value of 12.91 ± 0.56. They varied with pCO₂ or temperature according to size (Table 2, Figure 2). In small C. fornicata individuals, O:N ratios were the highest at 750 µatm and similar between 380 and
In large individuals, the O:N ratios varied with temperature and were significantly higher at 16°C.

_Filtration_

Temperature significantly affected filtration rates in both small and large individuals (Figure 1, Table 2). In small _C. fornicata_, mean filtration rates among pCO$_2$ were similar between 10 and 16°C (25.50 mL g$^{-1}$ DW min$^{-1}$), but increased at 19°C (54.30 mL g$^{-1}$ DW min$^{-1}$). pCO$_2$ alone did not affect the filtration rate but the interaction of pCO$_2$ and temperature was significant (Table 2, p-value < 0.001). At 19°C, filtration rates increased significantly with the increase in pCO$_2$. In large individuals, mean filtration rates increased gradually from 10°C (5.43 mL g$^{-1}$ DW min$^{-1}$) to 19°C (25.78 mL g$^{-1}$ DW min$^{-1}$) without any effect of pCO$_2$ conditions.

_Calcification_

Calcification rates were significantly affected by pCO$_2$ increase in both small and large individuals but not by temperature (Figure 1, Table 2). Pooling all temperature levels together, mean calcification rates were similar at pCO$_2$ of 390 µatm (1.88 and 1.63 µmol CaCO$_3$ g$^{-1}$ DW h$^{-1}$ in small and large individuals, respectively) and 750 µatm (1.02 and 0.60 µmol CaCO$_3$ g$^{-1}$ DW h$^{-1}$ in small and large, respectively), but significantly lower at 1400 µatm pCO$_2$ (-2.53 and -1.77 µmol CaCO$_3$ g$^{-1}$ DW h$^{-1}$ in small and large individuals, respectively). In the highest pCO$_2$ condition (1400 µatm), net calcification rates were negative, corresponding to dissolution. Although the interaction between pCO$_2$ and temperature was not significant for either small or large limpets, pCO$_2$ response appeared to vary as a function of temperature, particularly at 1400 µatm. In this drastic pCO$_2$ condition, organisms globally dissolved at 10, 13 and 16°C and calcified (or dissolved less) at 19°C.
Calcification rates decreased with the decrease in the mean aragonite saturation state ($\Omega_{Ar}$) which correlated with $pCO_2$ increase (Figure 3). When $\Omega_{Ar}$ decreased below the threshold of 1, calcification rates were always negative reflecting a dissolution process. At the 750 and 1400 µatm $pCO_2$ conditions, $\Omega_{Ar}$ was higher at 19°C than at the other temperature levels because the saturation state increases with temperature.

**Mesocosm controls**

In the aquaria maintained at 10°C throughout the entire experiment, temperature was stable over the first weeks of the experiment and slowly increased from 8 April to the end of the experiment until reaching a mean of 12.4°C between 21 April and 15 June because we had technical problems with the chiller (Table 1). Respiration in small individuals showed high variation over time (Figure 4, white bars) but no time effect was detected (Friedman test, $df = 3$, $\chi^2 = 6.6$, $p = 0.086$, $n = 6$). Conversely, respiration rates of large individuals increased throughout the experiment (Figure 4, gray bars) with a significant time effect (Friedman test, $df = 3$, $\chi^2 = 9.4$, $p = 0.024$, $n = 6$).

**Discussion**

An increase in temperature affected three of the four physiological processes assessed on small and large *C. fornicata* individuals. In particular, respiration and ammonia excretion rates clearly increased along the tested temperature gradient. In contrast, increases in $pCO_2$ affected only net calcification of the slipper limpets. Interestingly, the coupled effect of temperature and $pCO_2$ improved the rate of calcification in the most drastic conditions, particularly in small individuals.

**Temperature effect**
The respiration (0.6 - 34.6 µmol O$_2$ g$^{-1}$ DW h$^{-1}$) and excretion rates (-2 - 4.4 µmol NH$_3$ g$^{-1}$ DW h$^{-1}$) measured at 390 µatm pCO$_2$ in small and large *C. fornicata* individuals ranged metabolic rates recorded *in situ* in the Bay of Brest in northwestern France (4 to 45 µmol O$_2$ g$^{-1}$ DW h$^{-1}$ and 0.5 to 2.3 µmol NH$_3$ g$^{-1}$ DW h$^{-1}$; Martin et al. 2006). Both rates increased with temperature in small and large individuals regardless of pCO$_2$. Although respiration rates gradually increased with temperature in small *C. fornicata* individuals, they only increased from 10°C to 13°C, remaining stable at higher temperatures in large *C. fornicata*. This increase is a common response due to the rate-enhancing effects of temperature on physiological and biochemical reactions in ectotherms (Cossins and Bowler 1987). The intensity of respiratory and excretory processes were also dependent of body size. The respiration and excretion rates of small individuals were higher than those of large individuals because the metabolic rate (per unit biomass) decreases with increasing individual size (Parsons et al. 1984; Von Bertalanffy 1951). Small individuals have higher energy consumption because they grow faster than the large individuals (Von Bertalanffy 1964).

The filtration rates measured in small and large *C. fornicata* fall into the range of maximum feeding rates calculated by Newell and Kofoed (1977) in *C. fornicata* between 11 and 20°C (18 to 41 mL g$^{-1}$ min$^{-1}$; 15°C acclimated individuals). Rates were higher in small than in large individuals because, again, small organisms feed more actively per unit body mass (Sylvester et al. 2005). Filtration rates increased with temperature as previously described in other studies (Newell and Kofoed 1977). In small individuals, rates were constant between 10 and 16°C and increased only at 19°C while they increased regularly with temperature in the large individuals. In Calyptraeidae, small individuals — i.e. males with low mobility — utilize two feeding strategies: grazing with radula and filtration with gills (Navarro and Chaparro 2002). Therefore, small individuals may have supplemented their diet between 10 and 16°C by grazing. For the increased energy requirements at 19°C, small
slipper limpets may also increase their filtration rate to meet these supplementary needs. In large sedentary individuals (usually females), filtration is the only feeding mechanism (Navarro and Chaparro 2002) and filtration rate increases with temperature to help cover the higher energy needs.

Surprisingly, temperature did not affect calcification rates although an increase was expected in response to the increase in metabolism and energy requirements (Martin et al. 2006). Because mollusk shell production is an energetically costly process (Gazeau et al. 2013), the absence of any change in calcification rates may be due to food limitation during the experiment, especially at elevated temperatures (16 and 19°C). At these temperatures, providing additional food only twice a week may not have been sufficient to support maximal individual shell growth under pH stressful conditions. If food had been provided more regularly and/or in higher quantities, *C. fornicata* calcification may not be potentially restricted and individuals may have better mitigated the effect of high *pCO₂* (Thomsen et al. 2014). Future experiments should include measuring integrated shell growth at each temperature level to determine the food effect more completely.

Mesocosm experiments cannot perfectly reproduce *in situ* conditions such as natural diet or tidal cycles. This may lead to an increased stress for the organisms grown in these systems (Bibby et al. 2008). The mesocosm effect on organisms was tested through *O₂* consumption measurements in individuals kept at constant temperature throughout the experiment (“controls”). The respiration rates did not change over time in small individuals, whereas the respiration in large individuals increased slightly in correlation with a +2°C temperature increase from the beginning to the end of the experiment, because of technical problems with the chiller. Although food may have constituted a bias, particularly in the one-off calcification response to temperature, the absence of strong changes in respiration rates in “controls”, unexceptional metabolic rates ranging those measured *in situ* and very low
mortality during the experiment (only 8%) all suggest the absence of any acute mesocosm
effect on the other physiological traits of *C. fornicata*.

*pCO₂ effect*

In contrast to temperature, *pCO₂* did not affect *C. fornicata* respiration or excretion
rates regardless of size. Other studies have underlined a lack of any *pCO₂* effect on bivalve
and limpet respiration (Dickinson et al. 2012; Fernandez-Reiriz et al. 2012; Marchant et al.
2010), although some mollusk species exposed to high *pCO₂* levels have shown metabolic
depression (i.e. decrease in oxygen uptake) to compensate — albeit often drastic — *pCO₂*
increases (Michaelidis et al. 2005; Navarro et al. 2013). Responses of ammonia excretion to
high *pCO₂* in mollusks are also specific: increase in ammonia excretion can occur under
elevated *pCO₂* (Fernandez-Reiriz et al. 2011; Langenbuch and Pörtner 2002; Range et al.
2014) while some bivalves show opposing trends (Liu and He 2012; Navarro et al. 2013). The
increase in ammonia excretion under increased *pCO₂* conditions can be interpreted as an
internal pH regulatory mechanism, sometimes based on protein catabolism (Fernandez-Reiriz
et al. 2012; Thomsen and Melzner 2010). In our study, neither change in excretion rates nor in
O:N ratios calculated were detected between the 390 and 1400 µatm conditions. This
similarity indicates that potential intracellular pH regulation of *C. fornicata* was not induced
by enhancing protein metabolism (Fernandez-Reiriz et al. 2012). Thus, the potential for
metabolic resistance of *C. fornicata* to elevated *pCO₂* is likely due to another effective
acidosis-buffering system, such as the increase in internal HCO₃⁻ concentrations (Gutowska et
al. 2010; Michaelidis et al. 2005) or higher H⁺ excretion (Melzner et al. 2009; Pörtner et al.
2005).

Similarly to the respiration and excretion processes, filtration rates did not change as a
function of *pCO₂* in either small or large *C. fornicata* in our study. Filtration responses with
respect to $p\text{CO}_2$ depend most of the time on the presence of metabolic depression (Fernandez-Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013). The absence of variation in filtration rates at the different $p\text{CO}_2$ levels indicates that the quantity of food ingested by $C. \text{fornicata}$ did not vary either. Food is known to interact with other stressors, such as $p\text{CO}_2$, and significantly influence metabolic responses (Melzner et al. 2011; Pansch et al. 2014). Quality or quantity changes in the diet can even worsen the condition of invertebrates (Berge et al. 2006; Vargas et al. 2013). Although our microalgal mix did not perfectly match the natural diet of $C. \text{fornicata}$ (Barillé et al. 2006; Decottignies et al. 2007), the diatoms and dinoflagellate microalgae provided in the experiment correspond to the main taxa present in Morlaix Bay, assuming a nutritional quality close to the natural diet. However, we cannot assure that the quantity of food was not a limiting factor in our experiment. To be sure that microalgae supplied would not represent a bias, the slipper limpets should be fed *ad libitum* which represented a technical issue on a 6 month experiment.

In our study, net calcification was similar between 390 and 750 µatm $p\text{CO}_2$ and strongly decreased at 1400 µatm $p\text{CO}_2$ regardless of size, which is a common response in mollusks (Beniash et al. 2010; Melatunan et al. 2013; Range et al. 2011). This pattern contrasts with that reported in Ries et al. (2009), with a parabolic response in $C. \text{fornicata}$ calcification with the highest rates observed at 600 µatm $p\text{CO}_2$. The stability of calcification rate at 750 µatm $p\text{CO}_2$ (compared to 390 µatm $p\text{CO}_2$) may be due to the biological control of the calcification process and/or the presence of the periostracum, the organic layer covering the crystalline layers of the shell. This organic layer has been predicted to play a great role in maintaining shell integrity of mollusks in elevated $p\text{CO}_2$ conditions (Ries et al. 2009) and to protect them from dissolution in $\text{CaCO}_3$-undersaturated waters (Huning et al. 2013). Moreover, mollusks may be able to maintain extrapallial fluid in chemical conditions favoring $\text{CaCO}_3$ precipitation at the calcification site, even if external seawater $p\text{CO}_2$ is high.
Regulation of enzymes involved in the calcification process, such as chitinase (Cummings et al. 2011) or carbonic anhydrase (Ivanina et al. 2013), may also help maintain calcification in high $pCO_2$ conditions. In our study, at 1400 μatm, calcification rates dropped, perhaps due to physiological changes in the internal acid-base balance affecting shell deposition (Waldbusser et al. 2011) or to an eroded and/or damaged periostracum (pers. obs.). Degradation of this protective layer may lead to higher vulnerability of the shell to external dissolution processes (Range et al. 2012; Ries et al. 2009), which occurs not only in dead shells but also in live animals (Harper 2000). Furthermore, chemical dissolution increased with an increase in $pCO_2$ and a correlated decrease in $\Omega_{Ar}$; the combined effect led to a decrease in net calcification rates observed in both small and large *C. fornicata* individuals at high $pCO_2$ conditions.

### Combined effects of temperature and $pCO_2$

In the range of $pCO_2$ and temperatures tested, the interaction of these two variables had no negative effect on *C. fornicata* respiration and excretion rates. As a eurythermal species even coping with high temperature in some bays during summer (e.g. Bassin d’Arcachon in southwestern France; De Montaudouin et al. 1999), *C. fornicata* can have an optimal temperature of 19°C or higher (Diederich and Pechenik 2013; Noisette et al. 2015). Thus, 19°C may not constitute a real thermal stress and not transgress the metabolic optimal threshold for this species. Increase in temperature is predicted to enhance sensitivity to high $pCO_2$ levels beyond the optimal temperature of the species and close to its upper limit of thermal tolerance (Pörtner and Farrell 2008). However, at the cold side of a species optimal temperature, warming can increase resilience to ocean acidification (Gianguzza et al. 2014). Therefore, an increase in temperature may actually improve tolerance to $pCO_2$ increases in *C. fornicata*. 
Calcification rates of both small and large *C. fornicata* showed a positive trend with temperature in the most drastic $p$CO$_2$ conditions (1400 µatm). Temperature-mediated increases in metabolism and feeding rates may potentially offset reductions in calcification rates caused by high $p$CO$_2$ conditions (Melzner et al. 2011; Thomsen et al. 2014). In addition to this physiological effect, moderate warming can mediate the effects of ocean acidification by the chemical effect on seawater chemistry (Kroeker et al. 2014). Temperature affects CO$_2$ solubility in seawater as well as the equilibrium coefficients governing carbonate chemistry (Millero 2007). As shown in our study, the saturation state of aragonite was greater in warmer water than in colder water for a given $p$CO$_2$, thereby enhancing calcification and reducing the dissolution processes in the high $p$CO$_2$ conditions. These results highlight the importance of considering the physiological and geochemical interactions between temperature and carbonate chemistry when interpreting species’ vulnerability to ocean acidification. A better understanding of how warming influences species’ responses to high $p$CO$_2$ levels through both direct (e.g. increases in metabolic rates) and indirect pathways (e.g. changes in carbonate chemistry) is thus necessary.

**Conclusion**

A trade-off between stressors may affect the physiology of organisms in an unexpected way (Kroeker et al. 2014). In our case, *C. fornicata* appeared to be able to tolerate slight increases in $p$CO$_2$ but its calcification was affected by drastic conditions with a positive effect of temperature, thereby mitigating any ocean acidification effects. This outcome highlights the need of multistressor studies to understand the future of marine species in a context of climate change in which different physico-chemical factors vary in different ways. Furthermore, our results indicate that some species can be highly tolerant to future $p$CO$_2$ increases. *C. fornicata* tolerance likely stems from mechanisms that allow it to acclimate or
adapt to environmental fluctuations in its habitat (Clark et al. 2013), because species living in environments with large abiotic variations tend to have high phenotypic plasticity, allowing them to survive in stressful conditions (Somero 2010). This capacity to resist decreases in pH may reinforce the ecological role of C. fornicata populations in the ecosystems in which they are established, even under projected future conditions anticipated due to climate change.

References


Acknowledgments

The authors thank the “Marine Operations and Services Department” at the Station Biologique de Roscoff for the underwater sampling. We also thank the “Multicellular Marine Models” staff, and especially Ronan Garnier, for providing microalgae and their help for building the aquarium system. In addition, we are grateful to SOMLIT (Service d’Observation en Milieu LIToral, INSU-CNRS) for providing the abiotic parameter datasets. We are also grateful to Roseline Edern for her help with cytometry flux analysis. We really appreciated the editor and reviewers’ helpful and constructive comments which greatly improved this manuscript. This work was supported by the CALCAO project, which received funding from the Brittany Regional Council, and contributed to the “European Project on Ocean
Acidification” (EPOCA) funded by the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 211384. It was also supported by the Interreg IV a France (Channel) – England Marinexus project no. 4073 funded by the FEDER program.
Table 1: Mean seawater temperature and parameters of the carbonate system in each \( p\text{CO}_2 \) treatment (3 aquaria per treatment) and at each trial period (i.e. temperature level). The pH\(_T\) (pH on the total scale) and total alkalinity (A\(_T\)) were measured whereas the other parameters were calculated. Mean A\(_T\) calculated for each trial period (\( n = 3 \) for controls 10°C and 19 < \( n < 30 \) for other condition \( p\text{CO}_2 \) conditions) and \( p\text{CO}_2 \) condition was used for the calculations. \( p\text{CO}_2 \), CO\(_2\) partial pressure; DIC, dissolved inorganic carbon and \( \Omega_{\text{Ar}} \), saturation state of seawater with respect to aragonite.

<table>
<thead>
<tr>
<th>1(^{st}) trial period (10°C)</th>
<th>2(^{nd}) trial period (13°C)</th>
<th>3(^{rd}) trial period (16°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong> (°C)</td>
<td><strong>pH(_T)</strong> (µatm)</td>
<td><strong>pCO(_2)</strong> (µatm)</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>390 µatm</td>
<td>23</td>
<td>9.7</td>
</tr>
<tr>
<td>750 µatm</td>
<td>23</td>
<td>9.8</td>
</tr>
<tr>
<td>1400 µatm</td>
<td>23</td>
<td>9.5</td>
</tr>
<tr>
<td>control 10°C</td>
<td>40</td>
<td>9.2</td>
</tr>
<tr>
<td>390 µatm</td>
<td>28</td>
<td>15.9</td>
</tr>
<tr>
<td>750 µatm</td>
<td>28</td>
<td>16.1</td>
</tr>
<tr>
<td>1400 µatm</td>
<td>28</td>
<td>16.0</td>
</tr>
<tr>
<td>control 10°C</td>
<td>42</td>
<td>11.4</td>
</tr>
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</tr>
<tr>
<td>390 µatm</td>
<td>23</td>
<td>18.4</td>
</tr>
<tr>
<td>750 µatm</td>
<td>23</td>
<td>18.6</td>
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<tr>
<td>1400 µatm</td>
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<td>18.4</td>
</tr>
<tr>
<td>control 10°C</td>
<td>23</td>
<td>12.4</td>
</tr>
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</table>
Table 2: Summary of two-way repeated measurements ANOVAs followed by Student-Newman-Keuls post hoc tests testing the effect of $p$CO$_2$, temperature and their interaction on *Crepidula fornicata* physiology. Numbers in bold indicate significant p-values and values with different letters are significantly different at $p < 0.05$.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Two-way repeated measurements ANOVAs</th>
<th>Post hoc SNK tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p$CO$_2$</td>
<td>Temperature</td>
</tr>
<tr>
<td></td>
<td>df F p</td>
<td>df F p</td>
</tr>
<tr>
<td>Small individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiration</td>
<td>2 1.685 0.219 3 14.530 &lt; <strong>0.001</strong></td>
<td>6 1.893 0.103</td>
</tr>
<tr>
<td>Excretion</td>
<td>2 0.386 0.686 3 5.840 0.002</td>
<td>6 1.257 0.296</td>
</tr>
<tr>
<td>Filtration</td>
<td>2 0.271 0.766 3 15.439 &lt; <strong>0.001</strong></td>
<td>6 5.996 &lt; <strong>0.001</strong></td>
</tr>
<tr>
<td>Net calcification</td>
<td>2 6.705 <strong>0.008</strong> 3 1.849 0.152</td>
<td>6 2.307 <strong>0.050</strong></td>
</tr>
<tr>
<td>O:N ratio</td>
<td>2 4.944 <strong>0.022</strong> 2 2.214 0.127</td>
<td>4 0.382 0.819</td>
</tr>
<tr>
<td>Large individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiration</td>
<td>2 0.377 0.692 3 8.398 &lt; <strong>0.001</strong></td>
<td>6 0.523 0.788</td>
</tr>
<tr>
<td>Excretion</td>
<td>2 0.563 0.581 3 17.850 &lt; <strong>0.001</strong></td>
<td>6 0.371 0.893</td>
</tr>
<tr>
<td>Filtration</td>
<td>2 1.593 0.236 3 19.311 &lt; <strong>0.001</strong></td>
<td>0 2.012 0.083</td>
</tr>
<tr>
<td>Net calcification</td>
<td>2 13.615 &lt; <strong>0.001</strong> 3 0.878 0.459</td>
<td>6 0.911 0.496</td>
</tr>
<tr>
<td>O:N ratio</td>
<td>2 0.739 0.494 2 20.714 &lt; <strong>0.001</strong></td>
<td>4 1.728 0.170</td>
</tr>
</tbody>
</table>
**Figures**

**Figure 1:** Individual respiration, ammonia excretion, filtration and net calcification rates in the three $p\text{CO}_2$ treatments (shaded in grey) at 10, 13, 16 and 19°C for small (< 3 cm in length) and large (> 4.5 cm in length) *C. fornicata* individuals. Different letters above bars or before $p\text{CO}_2$ caption indicate significant differences between temperature or $p\text{CO}_2$ conditions, respectively. Results are expressed as mean ± standard error, n = 6 individuals.

**Figure 2:** O:N ratios for the three $p\text{CO}_2$ treatments (shaded in grey) at 13, 16 and 19°C for small and large *C. fornicata* individuals. Different letters above bars or before $p\text{CO}_2$ caption indicate significant differences between temperature or $p\text{CO}_2$ conditions, respectively. Results are expressed as mean ± standard error, n = 6 individuals.

**Figure 3:** Mean net calcification rates as function of aragonite saturation state, in the three $p\text{CO}_2$ treatments (shaded in grey), at 10 ($\bigcirc$), 13 ($\triangle$), 16 ($\square$) and 19°C ($\diamond$) for all *C. fornicata* individuals (n = 12 individuals).

**Figure 4:** Respiration rates in the control treatment (10°C) for the different trial periods (i.e. temperature levels) for single small (white bars) and large (grey bars) *C. fornicata* individuals. Results are expressed as mean ± standard error, n = 6 individuals.
Figure 1:

**Respiration (μmol O₂ g⁻¹ DW h⁻¹)**
- 390 μatm
- 750 μatm
- 1400 μatm

**Excretion (μmol NH₄⁺ g⁻¹ DW h⁻¹)**
- 390 μatm
- 750 μatm
- 1400 μatm

**Filtration (mL SW g⁻¹ DW min⁻¹)**
- 390 μatm
- 750 μatm
- 1400 μatm

**Net calcification (μmol CaCO₃ g⁻¹ DW h⁻¹)**
- 390 μatm
- 750 μatm
- 1400 μatm
Figure 2:

![Graph showing O:N ratio for small and large individuals across different temperatures and CO2 concentrations.]

Figure 3:

![Graph showing mean calcification rate (μmol CaCO3 g⁻¹ DW h⁻¹) against saturation state (Omega Ar).]
Figure 4:

**Small individuals**

![Graph showing respiration (µmol O₂ g⁻¹ DW h⁻¹) for Small individuals.]

**Large individuals**

![Graph showing respiration (µmol O₂ g⁻¹ DW h⁻¹) for Large individuals.]

Trial period