TITLE

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

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Abstract

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 pCO_2 conditions (390, 750 and 1400 µatm) at four successive temperature levels (10, 13, 16 and 19°C). At each temperature level and in each pCO_2 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 pCO_2 negatively affected calcification rates, leading to net dissolution in the most drastic pCO_2 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 pCO_2 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, pCO_2, 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 (pCO_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 (Ω), 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 pCO_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 (Beniash 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 19^{th} 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 pCO_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

149 measured with calipers, volume was estimated as the volume of seawater moved when
150 individual was immersed and DW was determined after drying fresh samples at 60°C for 48 h.
151
152 *Experimental conditions*
153 Single small and large individuals, along with their substratum shell, were randomly
154 distributed into nine 10 L aquaria with 10 individuals of each class size per each aquarium.
155 Empty shells were also distributed into nine other 10 L aquaria (4 shells per aquarium). At the
156 beginning of the experiment, pH was gradually decreased over 2 weeks by 0.02 pH unit per day
157 from 8.1 until the different pH treatments were reached. *C. fornicata* individuals and empty
158 shells were then subsequently held for 24 weeks (12 January to 27 June 2012) in three pCO_2
159 treatments selected according to the recommendations in Barry et al. (2010): (1) 390 µatm (pH_T
160 = 8.07) represented current pCO_2, (2) 750 µatm (pH_T = 7.82) corresponded to the elevated pCO_2
161 level predicted by the IPCC for the end of the century (Solomon et al. 2007) and (3) 1400 µatm
162 (pH_T = 7.56) represented a pCO_2 five-fold higher than preindustrial pCO_2 (280 µatm) also
163 predicted for 2100 (Stocker et al. 2013). pCO_2 was adjusted by bubbling CO_2-free air (current
164 pCO_2) or pure CO_2 (elevated pCO_2) in three 100 L header tanks supplied with unfiltered
165 seawater pumped directly from the foot of the *Station Biologique de Roscoff*. Each of the three
166 pCO_2 treatments had six replicate 10 L aquaria, three for live organisms and three for empty
167 shells. They continuously received CO_2-treated seawater at a rate of 9 L h^{-1} (i.e. a renewal rate
168 of 90% h^{-1}) from the header tanks. pCO_2 was monitored and controlled by an offline feedback
169 system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header
170 tanks. The pH values of the IKS system were adjusted from daily measurements of pH_T in the
171 18 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated
172 with Tris HCl and 2-aminopyridine HCl buffers (Dickson et al. 2007).

In each pCO_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^{st} trial period) from 16 January to 12 February 2012; (2) 13°C (2^{nd} trial period) from 27 February to 25 March 2012; (3) 16°C (3^{rd} trial period) from 9 April to 6 May 2012, and (4) 19°C (4^{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 pCO_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* (~ 15×10^6 cells mL^{-1}) and the dinoflagellate *Isochrysis affinis galbana* (~ 26×10^6 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.

198 Individuals that did not adhere to their substratum shell and that showed no reaction
199 when their foot was stimulated were counted as dead and removed from the tanks. Mortality
200 reached only 8% at the end of the experiment among all pCO_2 conditions.
201
202 *Seawater parameter monitoring*
203 Seawater parameters were monitored throughout the experiment. pH_T and temperature
204 were recorded daily in each of the 21 aquaria (18 + 3 controls) using a pH meter (826 pH
205 mobile, Metrohm AG, Herisau, Switzerland) as described above. Total alkalinity (A_T) was
206 measured at each trial period by 0.01 N HCl potentiometric titration on an automatic titrator
207 (Titroline alpha, Schott SI Analytics, Mainz, Germany). Salinity was also measured at each trial
208 period with a conductimeter (LF 330/ SET, WTW, Weilheim, Germany). Seawater carbonate
209 chemistry, i.e. dissolved inorganic carbon (DIC), pCO_2 and the saturation state of aragonite
210 (Ω_{Ar}) were calculated for each pCO_2 level and temperature with CO_2SYS software (Lewis and
211 Wallace 1998) using constants from Mehrbach et al. (1973) refitted by Dickson & Millero
212 (1987).
213
214 *Metabolic rates and O:N ratios*
215 Metabolic rates were assessed at each temperature level after a four-day starvation
216 period and after the shells were gently cleaned to remove biofilm-forming organisms. Two
217 small and two large individuals were selected per aquarium. They were incubated individually
218 in 185 mL (small) and 316 mL (large) acrylic chambers (Engineering & Design Plastics Ltd,
219 Cambridge, UK) filled with seawater from their respective aquaria. They were put on a plastic
220 grid above a stirring bar (speed 100 rpm.), which ensured water homogeneity. Chambers were
221 placed in their original aquaria for incubation to keep the temperature constant. Incubations
222 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 O_2 g^{-1} DW h^{-1}; equation [1]) and excretion (in µmol NH_4^+ g^{-1} DW h^{-1}; equation [2]) were directly calculated from oxygen and ammonium concentrations, respectively. Net calcification (in µmol $CaCO_2$ 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 $CaCO_3$ 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 $CaCO_3$ fluxes.

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

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

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

where ΔO_2 (in µmol O_2 L^{-1}) is the difference between initial and final O_2 concentrations; ΔNH_4^+ (in µmol NH_4^+ L^{-1}) is the difference between initial and final NH_4^+ concentrations; ΔA_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; Δt (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]: O:N = 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 pCO_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. 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 ± 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°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]:

$$[5] \quad F = V \times \frac{\ln[Ci] - \ln[Cf]}{\Delta t \times DW}$$

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. 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 pCO_2 increased from the baseline (+ 100-200 µatm). A_T ranged from 2365 ± 2 to 2422 ± 2 µEq kg^{-1}. Ω_{Ar} decreased by less than 1 only in the 1400 µatm pCO_2 condition. Salinity varied between 34.2 ± 0.1 and 35.1 ± 0.1 among the different pCO_2 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 pCO_2, in small and large individuals (Figure 1, Table 2). After pooling results for all pCO_2 conditions, mean respiration rates in small *C. fornicata* increased from 3.78 µmol O_2 g^{-1} DW h^{-1} at 10°C to 11.76 µmol O_2 g^{-1} DW h^{-1} at 19°C. In large individuals, the lowest mean respiration rate was recorded at 10°C (4.82 µmol O_2 g^{-1} DW h^{-1}) whereas rates did not differ from 13 to 19°C with a mean value of 11.50 µmol O_2 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 pCO_2 conditions for small *C. fornicata* individuals gradually increased from 0.15 µmol NH_3 g^{-1} DW h^{-1} at 10°C to 1.47 µmol NH_3 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 NH_3 g^{-1} DW h^{-1}) to 16°C (1.34 µmol NH_3 g^{-1} DW h^{-1}) followed by a decrease at 19°C (0.74 µmol NH_3 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 pCO_2 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 1400 µatm. 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}

346 ^{1}). pCO_2 alone did not affect the filtration rate but the interaction of pCO_2 and temperature was
347 significant (Table 2, p-value < 0.001). At 19°C, filtration rates increased significantly with the
348 increase in pCO_2. In large individuals, mean filtration rates increased gradually from 10°C (5.43
349 mL g^{-1} DW min^{-1}) to 19°C (25.78 mL g^{-1} DW min^{-1}) without any effect of pCO_2 conditions.
350
351 *Calcification*
352 Calcification rates were significantly affected by pCO_2 increase in both small and large
353 individuals but not by temperature (Figure 1, Table 2). Pooling all temperature levels together,
354 mean calcification rates were similar at pCO_2 of 390 µatm (1.88 and 1.63 µmol $CaCO_3$ g^{-1} DW
355 h^{-1} in small and large individuals, respectively) and 750 µatm (1.02 and 0.60 µmol $CaCO_3$ g^{-1}
356 DW h^{-1} in small and large, respectively), but significantly lower at 1400 µatm pCO_2 (-2.53 and
357 -1.77 µmol $CaCO_3$ g^{-1} DW h^{-1} in small and large individuals, respectively). In the highest pCO_2
358 condition (1400 µatm), net calcification rates were negative, corresponding to dissolution.
359 Although the interaction between pCO_2 and temperature was not significant for either small or
360 large limpets, pCO_2 response appeared to vary as a function of temperature, particularly at 1400
361 µatm. In this drastic pCO_2 condition, organisms globally dissolved at 10, 13 and 16°C and
362 calcified (or dissolved less) at 19°C.
363 Calcification rates decreased with the decrease in the mean aragonite saturation state
364 (Ω_{Ar}) which correlated with pCO_2 increase (Figure 3). When Ω_{Ar} decreased below the threshold
365 of 1, calcification rates were always negative reflecting a dissolution process. At the 750 and
366 1400 µatm pCO_2 conditions, Ω_{Ar} was higher at 19°C than at the other temperature levels
367 because the saturation state increases with temperature.
368
369 *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, χ^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, χ^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 pCO_2 (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 O_2 consumption measurements in individuals kept ca 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*.

pCO_2 effect

In contrast to temperature, pCO_2 did not affect *C. fornicata* respiration or excretion rates regardless of size. Other studies have underlined a lack of any pCO_2 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 pCO_2 levels have shown metabolic depression (i.e.

445 decrease in oxygen uptake) to compensate — albeit often drastic — pCO_2 increases
446 (Michaelidis et al. 2005; Navarro et al. 2013). Responses of ammonia excretion to high pCO_2
447 in mollusks are also specific: increase in ammonia excretion can occur under elevated pCO_2
448 (Fernandez-Reiriz et al. 2011; Langenbuch and Pörtner 2002; Range et al. 2014) while some
449 bivalves show opposing trends (Liu and He 2012; Navarro et al. 2013). The increase in
450 ammonia excretion under increased pCO_2 conditions can be interpreted as an internal pH
451 regulatory mechanism, sometimes based on protein catabolism (Fernandez-Reiriz et al. 2012;
452 Thomsen and Melzner 2010). In our study, neither change in excretion rates nor in O:N ratios
453 calculated were detected between the 390 and 1400 µatm conditions. This similarity indicates
454 that potential intracellular pH regulation of *C. fornicata* was not induced by enhancing protein
455 metabolism (Fernandez-Reiriz et al. 2012). Thus, the potential for metabolic resistance of *C.*
456 *fornicata* to elevated pCO_2 is likely due to another effective acidosis-buffering system, such as
457 the increase in internal HCO_3^- concentrations (Gutowska et al. 2010; Michaelidis et al. 2005)
458 or higher H^+ excretion (Melzner et al. 2009; Pörtner et al. 2005).
459 Similarly to the respiration and excretion processes, filtration rates did not change as a
460 function of pCO_2 in either small or large *C. fornicata* in our study. Filtration responses with
461 respect to pCO_2 depend most of the time on the presence of metabolic depression (Fernandez-
462 Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013). The absence of variation in filtration
463 rates at the different pCO_2 levels indicates that the quantity of food ingested by *C. fornicata* did
464 not vary either. Food is known to interact with other stressors, such as pCO_2, and significantly
465 influence metabolic responses (Melzner et al. 2011; Pansch et al. 2014). Quality or quantity
466 changes in the diet can even worsen the condition of invertebrates (Berge et al. 2006; Vargas et
467 al. 2013). Although our microalgal mix did not perfectly match the natural diet of *C. fornicata*
468 (Barillé et al. 2006; Decottignies et al. 2007), the diatoms and dinoflagellate microalgae
469 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 pCO_2 and strongly decreased at 1400 µatm pCO_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. fornicata* calcification with the highest rates observed at 600 µatm pCO_2. The stability of calcification rate at 750 µatm pCO_2 (compared to 390 µatm pCO_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 pCO_2 conditions (Ries et al. 2009) and to protect them from dissolution in $CaCO_3$-undersaturated waters (Huning et al. 2013). Moreover, mollusks may be able to maintain extrapallial fluid in chemical conditions favoring $CaCO_3$ precipitation at the calcification site, even if external seawater pCO_2 is high (Hiebenthal et al. 2013). 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

494 in Ω_{Ar}; the combined effect led to a decrease in net calcification rates observed in both small
495 and large *C. fornicata* individuals at high pCO_2 conditions.
496
497 *Combined effects of temperature and pCO_2*
498 In the range of pCO_2 and temperatures tested, the interaction of these two variables had
499 no negative effect on *C. fornicata* respiration and excretion rates. As a eurythermal species even
500 coping with high temperature in some bays during summer (e.g. Bassin d'Arcachon in
501 southwestern France; De Montaudouin et al. 1999), *C. fornicata* can have an optimal
502 temperature of 19°C or higher (Diederich and Pechenik 2013; Noisette et al. 2015). Thus, 19°C
503 may not constitute a real thermal stress and not transgress the metabolic optimal threshold for
504 this species. Increase in temperature is predicted to enhance sensitivity to high pCO_2 levels
505 beyond the optimal temperature of the species and close to its upper limit of thermal tolerance
506 (Pörtner and Farrell 2008). However, at the cold side of a species optimal temperature, warming
507 can increase resilience to ocean acidification (Gianguzza et al. 2014). Therefore, an increase in
508 temperature may actually improve tolerance to pCO_2 increases in *C. fornicata*.
509 Calcification rates of both small and large *C. fornicata* showed a positive trend with
510 temperature in the most drastic pCO_2 conditions (1400 µatm). Temperature-mediated increases
511 in metabolism and feeding rates may potentially offset reductions in calcification rates caused
512 by high pCO_2 conditions (Melzner et al. 2011; Thomsen et al. 2014). In addition to this
513 physiological effect, moderate warming can mediate the effects of ocean acidification by the
514 chemical effect on seawater chemistry (Kroeker et al. 2014). Temperature affects CO_2 solubility
515 in seawater as well as the equilibrium coefficients governing carbonate chemistry (Millero
516 2007). As shown in our study, the saturation state of aragonite was greater in warmer water
517 than in colder water for a given pCO_2, thereby enhancing calcification and reducing the
518 dissolution processes in the high pCO_2 conditions. These results highlight the importance of

519 considering the physiological and geochemical interactions between temperature and carbonate
520 chemistry when interpreting species' vulnerability to ocean acidification. A better
521 understanding of how warming influences species' responses to high pCO_2 levels through both
522 direct (e.g. increases in metabolic rates) and indirect pathways (e.g. changes in carbonate
523 chemistry) is thus necessary.
524
525 *Conclusion*
526 A trade-off between stressors may affect the physiology of organisms in an unexpected
527 way (Kroeker et al. 2014). In our case, *C. fornicata* appeared to be able to tolerate slight
528 increases in pCO_2 but its calcification was affected by drastic conditions with a positive effect
529 of temperature, thereby mitigating any ocean acidification effects. This outcome highlights the
530 need of multistressor studies to understand the future of marine species in a context of climate
531 change in which different physico-chemical factors vary in different ways. Furthermore, our
532 results indicate that some species can be highly tolerant to future pCO_2 increases. *C. fornicata*
533 tolerance likely stems from mechanisms that allow it to acclimate or adapt to environmental
534 fluctuations in its habitat (Clark et al. 2013), because species living in environments with large
535 abiotic variations tend to have high phenotypic plasticity, allowing them to survive in stressful
536 conditions (Somero 2010). This capacity to resist decreases in pH may reinforce the ecological
537 role of *C. fornicata* populations in the ecosystems in which they are established, even under
538 projected future conditions anticipated due to climate change.
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Tables

Table 1: Mean seawater temperature and parameters of the carbonate system in each pCO_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 pCO_2 conditions) and pCO_2 condition was used for the calculations. pCO_2, CO_2 partial pressure; DIC, dissolved inorganic carbon and Ω_{Ar}, saturation state of seawater with respect to aragonite.

		Temperature (°C)		**pH_T**		**pCO_2** (µatm)		**A_T** (µEq kg^{-1})		**DIC** (µmol C kg^{-1})		**Ω_{Ar}**	
	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1st trial period (10°C)													
390 µatm	23	9.7	0.2	8.14	0.01	322	7	2365	2	2138	4	2.47	0.04
750 µatm	23	9.8	0.2	7.82	0.01	729	19	2368	2	2270	4	1.33	0.03
1400 µatm	23	9.5	0.2	7.55	0.03	1486	75	2376	2	2366	11	0.78	0.08
control 10°C	40	9.2	0.2	8.19	0.02	288	17	2370	3	2115	8	2.73	0.07
2nd trial period (13°C)													
390 µatm	27	12.9	0.2	8.12	0.02	356	25	2418	2	2167	8	2.76	0.07
750 µatm	27	13.0	0.1	7.81	0.01	781	20	2416	2	2304	3	1.48	0.03
1400 µatm	27	12.8	0.1	7.53	0.01	1557	43	2422	2	2405	4	0.82	0.02
control 10°C	41	11.0	0.1	8.18	0.01	297	12	2419	2	2152	5	2.88	0.05
3rd trial period (16°C)													
390 µatm	28	15.9	0.1	8.08	0.01	376	10	2379	5	2126	5	2.80	0.05
750 µatm	28	16.1	0.1	7.82	0.00	748	8	2369	5	2238	2	1.66	0.01

1400 µatm	28	16.0	0.1	7.55	0.01	1492	19	2380	5	2345	2	0.94	0.01
control 10°C	42	11.4	0.1	8.23	0.01	253	6	2376	4	2083	5	3.13	0.05

4th trial period (19°C)

390 µatm	23	18.4	0.5	8.02	0.01	450	10	2391	2	2152	5	2.70	0.05
750 µatm	23	18.6	0.5	7.77	0.01	858	19	2395	3	2266	4	1.68	0.04
1400 µatm	23	18.4	0.5	7.51	0.01	1652	41	2394	2	2359	4	0.96	0.03
control 10°C	23	12.4	0.1	8.20	0.01	280	12	2393	1	2107	8	3.07	0.08

Table 2: Summary of two-way repeated measurements ANOVAs followed by Student-Newman-Keuls post hoc tests testing the effect of pCO_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 $p < 0.05$.

	Two-way repeated measurements ANOVAs									**Post hoc SNK tests**						
	Factors									Factors						
	pCO_2			Temperature			pCO_2 x Temperature			pCO_2 (µatm)			Temperature (°C)			
	df	F	p	df	F	p	df	F	p	390	750	1400	10	13	16	19
Small individuals																
Respiration	2	1.685	0.219	3	14.530	**< 0.001**	6	1.893	0.103				a	b	b	c
Excretion	2	0.386	0.686	3	5.840	**0.002**	6	1.257	0.296				a	a,b	b	b
Filtration	2	0.271	0.766	3	15.439	**< 0.001**	6	5.996	**< 0.001**				a	a	a	b
Net calcification	2	6.705	**0.008**	3	1.849	0.152	6	2.307	**0.050**	a	a	b				
O:N ratio	2	4.944	**0.022**	2	2.214	0.127	4	0.382	0.819	a	b	a				
Large individuals																
Respiration	2	0.377	0.692	3	8.398	**< 0.001**	6	0.523	0.788				a	b	b	b
Excretion	2	0.563	0.581	3	17.850	**< 0.001**	6	0.371	0.893				a	b	c	b
Filtration	2	1.593	0.236	3	19.311	**< 0.001**	0	2.012	0.083				a	b	b	c
Net calcification	2	13.615	**< 0.001**	3	0.878	0.459	6	0.911	0.496	a	a	b				
O:N ratio	2	0.739	0.494	2	20.714	**< 0.001**	4	1.728	0.170				-	a	b	a

Figures

Figure 1: Individual respiration, ammonia excretion, filtration and net calcification rates in the three pCO_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 pCO_2 caption indicate significant differences between temperature or pCO_2 conditions, respectively. Results are expressed as mean ± standard error, n = 6 individuals.

Figure 2: O:N ratios for the three pCO_2 treatments (shaded in grey) at 13, 16 and 19°C for small and large *C. fornicata* individuals. Different letters above bars or before pCO_2 caption indicate significant differences between temperature or pCO_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 pCO_2 treatments (shaded in grey), at 10 (○), 13 (△), 16 (□) and 19°C (◇) 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.

30 Figure 1 :

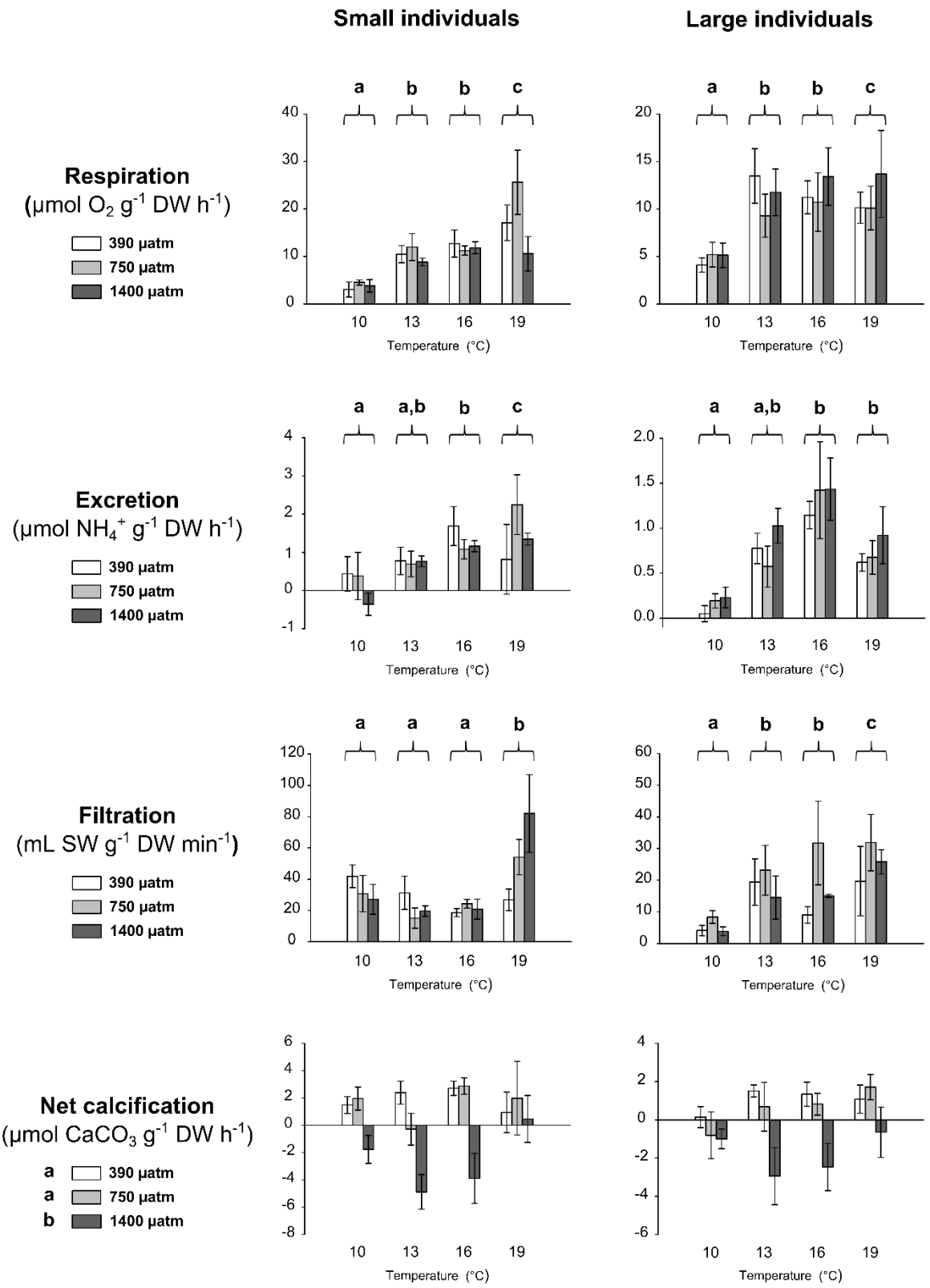

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Figure 2:

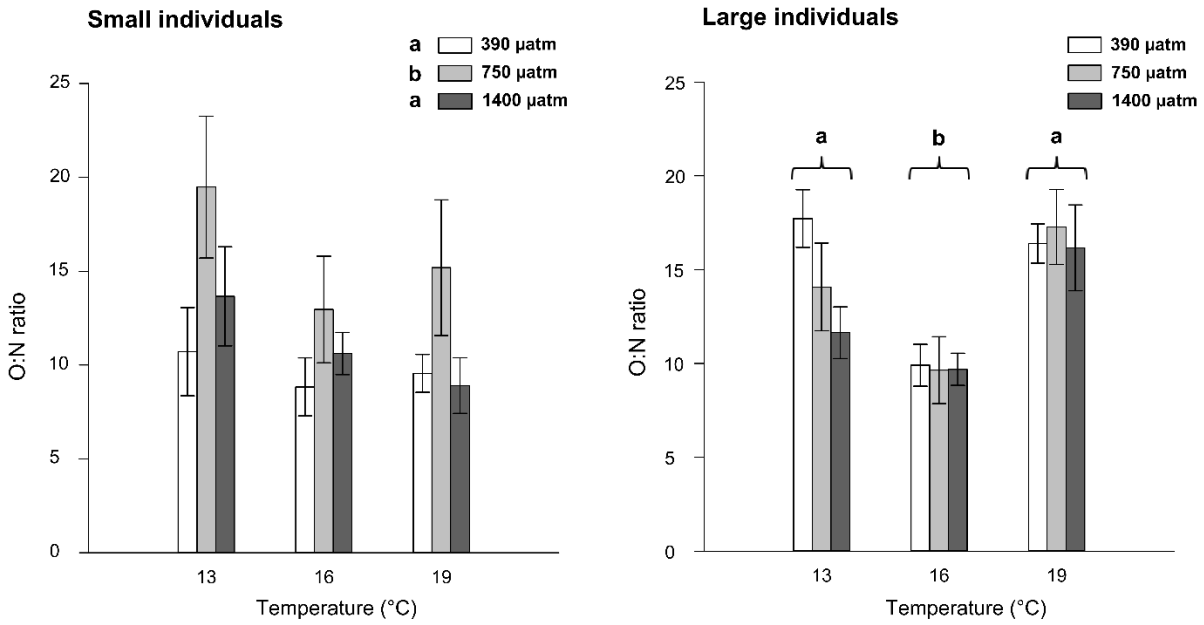


Figure 3:

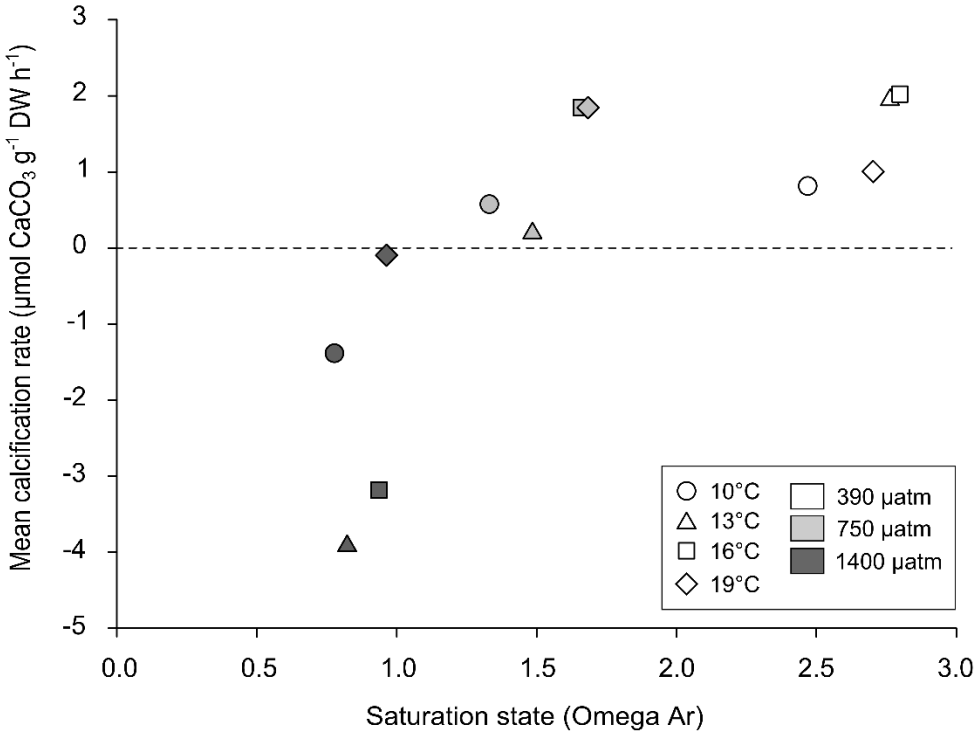
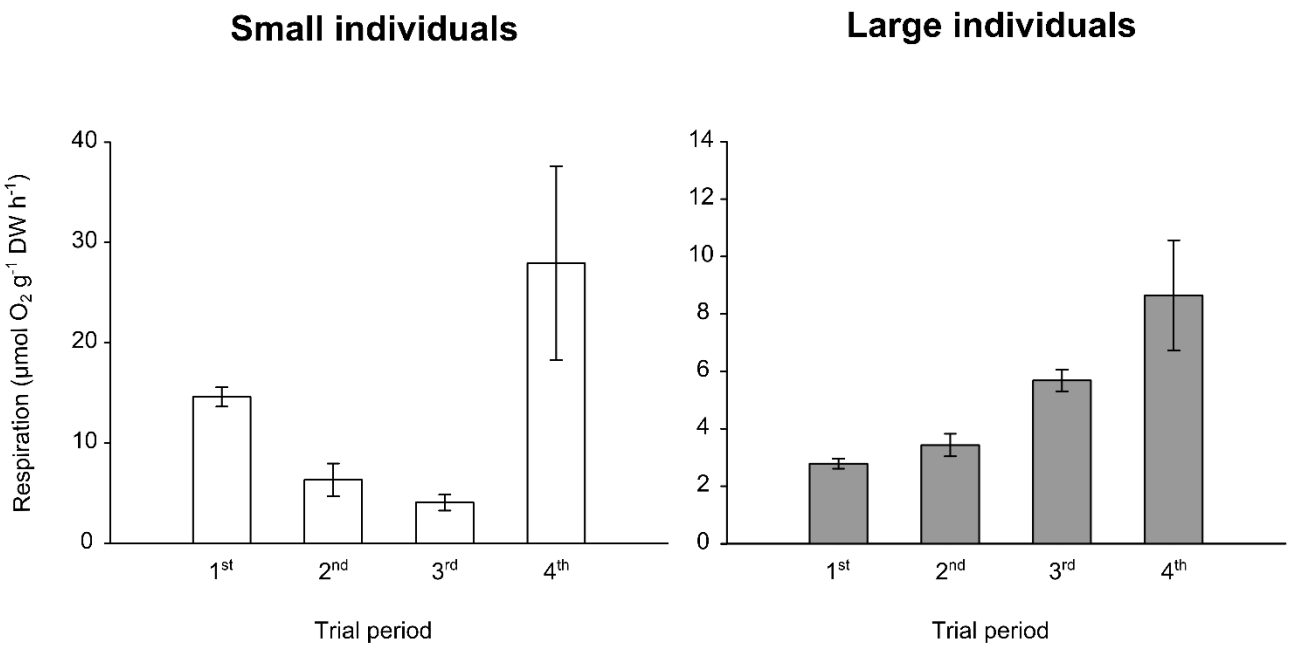


Figure 4:


Small individuals
Large individuals
Respiration (µmol O2 g-1 DW h-1)
40
30
20
10
0
14
12
10
8
6
4
2
0
1st
2nd
3rd
4th
Trial period
Trial period