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# Effect of the endosymbiotic pea crab *Calyptraeotheres garthi* on the metabolic rate and oxidative status of the slipper limpet *Crepidula cachimilla*

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**Abstract.** Parasites may induce metabolic changes and imbalances in the redox status of hosts. This study tested the effect of parasites on the $O_2$ consumption rate ($O_2$-CR) of hosts, and explored the link between $O_2$-CR and oxidative stress in parasitized hosts. We used the symbiotic pea crab *Calyptraeotheres garthi* and its slipper limpet host *Crepidula cachimilla* as models. The $O_2$-CR of long-term (3 months) infested limpets was 2.5 times greater than that of long-term uninfested limpets. Also, the $O_2$-CR of limpets stripped of crabs 24 h before measurements was intermediate between that of long-term infested and uninfested limpets. These results indicate a parasitic relationship between *C. garthi* and *Cr. cachimilla*, and suggest that the effect of the parasite on the metabolic rate of limpets is reversible. Lastly, the activity of two antioxidant enzymes (CAT and GST) as well as lipid peroxidation did not vary between infested and uninfested limpets. Thus, increased $O_2$-CR is not necessarily coupled with oxidative stress in pea crab-parasitized slipper limpets.

*Additional key words:* oxidative stress, oxygen consumption, Pinnotheridae

Parasitic relationships are ubiquitous in marine, freshwater, and terrestrial environments (Margulis & Fester 1991). Theoretical and empirical studies suggest that the negative effect(s) of parasites (with parasitism defined *sensu* Paracer & Ahmadjian [2000] as a partnership between individuals from different species in which one species, the parasite, benefits at the expense of the other, the host) are modulated via their impact on the host's energy budget (Lettini & Sukhdeo 2010; Robar et al. 2011). Parasites may affect host resource acquisition directly by disturbing the feeding behavior (Munger & Karasov 1989) or assimilation efficiency of host individuals (Moore 2002). Parasites can also affect the host energy budget indirectly by inducing immune responses, tissue repair and regeneration, or behavioral changes. These activities often result in substantial increases to the host's energy expenditure (Giorgi et al. 2001; Klasing 2004).

If parasites affect the metabolic rate of their host, shifts in the oxidative status of infested host individuals are also expected. Increases in oxygen consumption rate ($O_2$-CR) are known to induce oxidative stress (defined *sensu* Valavanidis et al. [2006] as the imbalance between the generation and neutralization of reactive oxygen species [ROS] by antioxidant mechanisms), because ROS are a by-product of $O_2$ metabolism in all aerobic organisms (Lushchak 2011; van de Crommenacker et al. 2012). Under high $O_2$ concentration, the incomplete reduction of $O_2$ in mitochondria results in the production of ROS that can oxidize lipids, proteins, and nucleic acids, and can lead to disease and aging (e.g., Lushchak 2011). To prevent cell damage, however, aerobic organisms have evolved antioxidant defense mechanisms capable of intercepting and inactivating ROS. These antioxidant defenses include the

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production of non-enzymatic (e.g., glutathione, vitamins A and E) and enzymatic (e.g., superoxide dismutase [SOD], catalase [CAT], peroxidases [GPx and AsPx], and glutathione S–transferase [GST]) mechanisms.

Only a few studies have explored the relationship between $O_2$-CR and oxidative stress markers in parasitic relationships (Di Giulio et al. 1995; Neves et al. 2000; Chambon et al. 2007). In some of these parasite-host pairs, host oxidative stress has been suggested to be the consequence of parasites that directly or indirectly increase the $O_2$-CR of their hosts (Neves et al. 2000). However, $O_2$-CR and oxidative stress markers have only rarely been measured together in parasitized hosts. One example is Chambon et al. (2007), who observed that the parasitic polychaete *Polydora* sp. increased arterial $PO_2$ in the oyster *Crassostrea gigas* (Thunberg 1793), and suggested that this physiological change led to oxidative stress in this host.

Pinnotheridae (i.e., pea crabs) is a family of brachyuran crabs that establish symbiotic associations (symbiosis defined *sensu* de Bary [1879] as dissimilar organisms living together) with a wide diversity of marine invertebrates (Schmitt et al. 1973). Pea crabs, particularly those inhabiting mollusks, are known to diminish growth rate (Bierbaum & Ferson 1986), feeding and metabolic rate (Bierbaum & Shumway 1988), and reproductive activity (e.g., brooding capacity: Chaparro et al. 2001) of their host individuals. The pea crab *Calyptraeotheres garthi* (Fenucci 1975) inhabits the mantle cavity of several species of slipper limpets in the southwestern Atlantic (Campos 1999), including *Crepidula cachimilla* Cledón, Simone & Penchaszadeh 2004, a species endemic to San Matias Gulf, Patagonia (Ocampo et al. 2012a). Although formerly considered to be a commensal (e.g., Campos 1999) rather than a parasite, Chaparro et al. (2001) demonstrated that a congener of *C. garthi*, *Calyptraeotheres* sp., castrates their limpet hosts. Examining the metabolic rate and oxidative status of *Cr. cachimilla* with and without pea crab symbionts will help demonstrate if *C. garthi* is also a parasite of slipper limpets.

The aim of this study was to determine whether or not the pea crab *C. garthi* affects the metabolic rate of the slipper limpet *Cr. cachimilla*. For this purpose, we examined the metabolic effect of pea crabs in short- and long-term crab-infested limpets, and tested whether or not the effect of pea crabs on the metabolism of limpets was reversible. Finally, if oxygen consumption rates did increase in infested compared with uninfested limpets, then we predicted that parasite-induced metabolic costs would affect the oxidative status of the infested limpets. To test this hypothesis, we measured the activity of CAT and GST as well as the content of lipid peroxidation in the limpet's gills.

## Methods

### Collection and maintenance of hosts and crabs

Specimens of *Crepidula cachimilla* were collected in September 2010 from the subtidal at Piedras Coloradas (40°57′S, 65°06′W), San Matias Gulf, North Patagonia, Argentina. *Crepidula cachimilla* inhabits depths between 10 and 40 m and is usually found attached to the valves of the mussel *Mytilus edulis* Linnaeus 1758 (Cledón et al. 2004). Slipper limpets were collected using dredges deployed at ~30 m depth from a fishing boat, placed in containers with aerated seawater, and transported alive to the Laboratory of Marine Ecology and Genetics (National University of Mar del Plata, Argentina).

The shell length (SL) of each limpet was measured with digital calipers to the nearest 0.1 mm. Because *Cr. cachimilla* are protandric hermaphrodites that changes sex at ~17.5 mm SL (Cledón et al. 2004) and the prevalence of *Calyptraeotheres garthi* is highest in limpets 32–38 mm in SL (Ocampo et al. 2012b), we selected only female limpets of 32–38 mm SL for our experiments.

After being measured and sexed, each limpet was detached from its substratum and carefully inspected for pea crabs, which are found between the ventral side of the neck and an anterior fold of the limpet's foot (Fig. 1). Pea crabs were sexed based on the presence or absence of gonopods (McLaughlin 1980). Only mature female crabs were returned to the same limpet from which they were originally extracted. We used only limpets harboring mature female crabs for our experiments as we expected a sex-specific effect of *C. garthi* on the metabolic rate and oxidative stress of their host individuals because (1) females but not necessarily males establish a long-term association with their host individuals, and (2) females attain much larger body sizes than males (Ocampo et al. 2012b). The relatively small male crabs appear to shift rather frequently among host individuals, and their effect on limpets might be minimal compared with that of the relatively large and sedentary female crabs (see Ocampo et al. 2012b). Finally, all experimental limpets were reattached to new substrates consisting of small flexible pieces of transparent plastic (see Ocampo et al. 2012b). This new substrate permitted us to visually determine whether a limpet was harboring a crab

**Fig. 1.** Ventral view of a limpet host (*Crepidula cachimilla*), and a female crab (*Calyptraeotheres garthi*) inside the limpet's pallial cavity (arrow) and covered in part by a fold of the foot. F, foot; G, gill; H, head; N, neck. Scale bar=5 mm.

and to introduce or remove a crab from within the limpet's pallial cavity without having to detach the host individual for a second time.

Prior to experiments, limpets were marked with epoxy paint applied on to the shell, and placed in a 300-L plastic stock tank with flowing fresh seawater (6–10 L $min^{-1}$) at 18–20°C and 34 PSU during a 3-month period. Limpets were observed daily. Female crabs were never observed to abandon or shift among different host individuals. In the stock tank, $PO_2$ was maintained at normoxic levels (water $PO_2$=21 kPa) by bubbling air constantly, and temperature, salinity, and $PO_2$ were monitored every 2–3 d. Limpets were fed daily with the unicellular algae *Nannochloropsis oculata* D.J. Hibberd 1981 and *Tetraselmis suecica* Butcher 1959. Both limpets and crabs eat these algae; limpets filter and feed on the algae while crabs feed on phytoplankton-rich mucus cords produced by limpets (Ocampo, unpubl. data).

### The effect of pea crabs on limpet metabolic rate

Because there is a well-established relationship between metabolism and oxygen consumption (Burggren & Roberts 1991), we used $O_2$ consumption rate ($O_2$-CR) as a proxy for the energetic costs experienced by limpet individuals when harboring symbiotic pea crabs.

A total of five different treatments were employed to test whether or not pea crabs affect the metabolic rate of host limpets (Table 1). In the first treatment (T-1: long-term infested limpet), limpets hosting female pea crabs when originally collected retained their crabs during the acclimatization period of 3 months. In the second treatment (T-2: short-term infested limpet), a single female pea crab was introduced into the pallial cavity of limpets harboring no female crabs when collected. Pea crabs were gently introduced into the host's pallial cavity 24 h before oxygen consumption measurements were recorded via a gap created between the limpet's body and the plastic piece of substrate by momentarily flexing the plastic. In the third treatment (T-3: short-term uninfested limpet), limpets hosting female pea crabs when originally collected were stripped of crabs 24 h before oxygen consumption measurements. The crab was removed from the host's pallial cavity while momentarily flexing the limpet's plastic sub-

**Table 1.** Details of morphometric measures of limpets and crabs used in the experiments.

| | N | Limpet shell length (mm) | Limpet fresh weight (g) | Limpet CI | Crab carapace width (mm) | Crab fresh weight (g) |
|---|---|---|---|---|---|---|
| Oxygen consumption experiment | | | | | | |
| T-1: long-term infested limpet | 6 | 37.50±1.65 | 3.56±0.62 | 5.35±0.42 | 6.08±0.37 | 0.082±0.012 |
| T-2: short-term infested limpet | 6 | 37.68±0.82 | 3.47±0.48 | 5.40±0.37 | 5.82±0.53 | 0.097±0.034 |
| T-3: short-term uninfested limpet | 5 | 37.58±1.39 | 3.98±0.50 | 5.38±0.42 | 6.15±0.51 | 0.077±0.027 |
| T-4: long-term uninfested limpet | 6 | 36.30±1.57 | 3.45±0.66 | 5.11±0.62 | — | — |
| T-5: non-infested limpet+crab | 5 | 37.74±2.23 | 3.81±0.77 | 5.46±0.68 | 6.10±0.33 | 0.092±0.019 |
| Enzymatic activity and lipid peroxidation experiment | | | | | | |
| T-1: long-term infested limpet | 5 | 37.46±1.38 | 3.53±0.64 | 5.32±0.64 | 6.30±0.41 | 0.102±0.033 |
| T-2: short-term infested limpet | 6 | 35.80±2.09 | 3.52±0.78 | 5.38±0.63 | 6.25±0.37 | 0.102±0.030 |
| T-3: long-term uninfested limpet | 6 | 34.80±2.63 | 3.61±0.96 | 5.35±0.73 | — | — |

CI, condition index; N, number of individuals; T, treatments.
Values represent mean±SD.

strate. In the fourth treatment (T-4: long-term uninfested limpet), a control, limpets did not harbor female pea crabs when originally collected, and no crabs were introduced into their pallial cavities. Lastly, in the fifth treatment (T-5: uninfested limpet+crab), we measured oxygen consumption in limpets not harboring female pea crabs when originally collected, together with an isolated female pea crab in the same respiratory chamber. After $O_2$-CR measurements in limpets from treatments T-1, T-2, and T-5, pea crabs were extracted from these limpet hosts and the $O_2$-CR of these isolated crabs was also measured. The individual $O_2$-CR values (i.e., the $O_2$ consumption rate expressed per individual) for limpets containing pea crabs were calculated by subtracting the individual $O_2$-CR values for isolated crabs from the initial readings, which represented the combined oxygen uptake of the limpet plus crab. $O_2$-CR values of limpets were expressed as mg of $O_2$ per g of fresh mass. The number of replicates per treatment was 5 or 6, depending upon limpet availability (Table 1).

In each treatment, $O_2$-CR was measured using an $O_2$-sensing system consisting of a fiber optic oxygen transmitter (Fibox 3) and a $O_2$-sensitive chemical optical sensor (PSt3: PreSens, Regensburg, Germany). This system is capable of non-intrusive high-resolution measurements of dissolved $O_2$ (Okubo et al. 2008). Experiments were conducted using a hermetically sealed cylindrical respiration chamber of 200 mL capacity (3 cm radius×7 cm height) submerged in a water bath (the lid of the chamber was just below the surface). Three different openings in the lid of the respiration chamber were traversed by two glass tubes and a holder of the $O_2$-sensitive optical sensor. One of the glass tubes was connected to the respiration chamber and was used to aerate the water to maintain the $O_2$ concentration near saturation within the respiration chamber prior to measurements. The second glass tube was used to decompress the respiration chamber from pressure generated by the entry of air from the first tube. Both tubes were hermetically sealed at the beginning of each replicate. The $O_2$-sensitive optical sensor used to monitor oxygen availability within the respiratory chamber had a tip diameter of ~2 mm and was calibrated (0% air saturation: solution saturated with 0.01 mol $L^{-1}$ $Na_2SO_3$; 100% air saturation: air–saturated filtered water) at 20°C. The $O_2$-sensitive optical sensor was inserted through the third lid hole and the tip of the optic fiber was fixed ~2 mm from the bottom of the respiratory chamber. Prior to the experiment, each limpet was gently cleaned of epibionts using a small knife. Experimental limpets and crabs were then introduced into the respiratory chamber filled with Millipore filtered seawater (mesh size 0.21 μm) at 20°C and 34 PSU. After a 30-min period of acclimation, the aeration was stopped and the chamber sealed. Oxygen partial pressure (% air saturation) was recorded every 10 s during an experimental period that lasted ~2 h. The experiment was run up to the point in which experimental limpets (and/or crabs) reduced the dissolved $O_2$ concentration by ~20% within the respiratory chamber. For each replicate, parallel measurements of $O_2$ concentration were conducted in a control chamber containing filtered seawater but no limpets and/or crabs. During the experiment, the water was circulated every 10 min within the respiratory chambers by gently moving one of the glass tubes. This maintained a uniform concentration of dissolved $O_2$ within both control and experimental chambers. All measurements were conducted during the afternoon (1400–1800 h). Oxygen concentration in the experimental chamber was corrected according to changes (if any) in the control chamber. $O_2$-CR was estimated as the slope of the least-squares regression line ($R^2$>0.9) showing change in $O_2$ tension in the chamber through time (mg $O_2$ $h^{-1}$ $g^{-1}$).

At the end of the experiment, limpets and/or crabs were weighed (fresh weight) with an analytical balance (precision=0.01 mg), and the shell length of each limpet and the carapace width (CW) of each crab were measured with digital calipers to the nearest 0.1 mm (Table 1). Because of the high variability in the shell shape of slipper limpets, conventional condition indices (CI) which employ shell volume could not be used (see Thain 1984); instead, the fresh weight of the soft body parts divided by the total fresh weight (soft body parts+shell)×100 was used as an index of condition (Narvarte & Saiz 2004). No differences in CI were found among limpets from the different treatments used in this experiment (one-way ANOVA, $F_{4,23}$=0.406, p=0.802). No differences in fresh weight were found among crabs used in this experiment (one-way ANOVA, $F_{4,23}$ = 0.665, p=0.623).

#### Limpet enzyme activities and lipid peroxidation

To determine whether changes in limpet $O_2$-CR led to oxidative stress in limpets, lipid peroxidation (LPO) and the activity of two antioxidant enzymes (catalase [CAT] and glutathione S–transferase [GST]) were examined. We measured proxies of oxidative stress in limpets from three different treatments: T-1 (long-term infested limpets), T-2 (short-term infested limpets), and T-3 (long-term

uninfested limpets) (for details of treatments, see above). The number of replicates per treatment was 5 or 6 depending upon limpet availability (Table 1). Limpets were euthanized by placing them on ice for 1 h, then dissected to obtain gill tissue that was immediately flash-frozen with liquid nitrogen and stored at −80°C until its processing.

Cytosolic enzymes were extracted from gill tissue following Wiegand et al. (2000), but without the purification step. Briefly, 50 mg of frozen gill tissue was homogenized using a Potter glass homogenizer (maintained in ice) with 5 mL sodium–phosphate buffer (0.1 mol $L^{-1}$, pH 6.5) containing 20% glycerol, 14 mmol $L^{-1}$ 1,4-dithioerythritol (DTE), and 1 mmol $L^{-1}$ EDTA. Next, debris was removed by centrifugation at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 1 h to separate microsomes from the soluble fraction. The cytosolic fraction was immediately flash-frozen using liquid nitrogen, and stored at −80°C until enzymatic activity was measured.

Enzymatic activity was determined with spectrophotometry. GST and CAT activities were determined following Claiborne (1985) and Habig et al. (1974), respectively. For GST activity, we used 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Each enzyme assay was carried out in duplicate. The total protein content of each sample was assessed spectrophotometrically by the Bradford (1976) method, using bovine serum albumin solution as standard. Enzyme activities are reported in nanokatals per mg of protein (nkat $mg^{-1}$ prot), where 1 katal corresponds to the conversion of 1 mol of substrate per second.

Lipid peroxidation in gill tissue was determined by measuring the formation of thiobarbituric acid reactive substances (TBARs) following Oakes & van der Kraak (2003). Briefly, 50 mg of tissue was homogenized in 450 μL of a solution of 1.15% KCl and butylated hydroxytoluene (BHT) at 35 mmol $L^{-1}$. Extracts (100 μL) were added to a reaction mixture made with 3 mL of 0.8% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA), 500 μL of Milli Q water, 200 μL of 8.1% SDS, and 200 μL of 1407 mmol $L^{-1}$ BHT (in ethanol). Next, samples were heated at 95°C for 30 min and then cooled down at room temperature for 10 min. After centrifugation (3000 *g* for 10 min at 15°C), the supernatant containing TBARs was measured with a spectrophotometer ($\lambda_{abs}$=532 nm). TBARs content was expressed as nanomoles per mg of fresh tissue, using a molar extinction coefficient of $1.56\times10^5$ $M^{-1}$ $cm^{-1}$.

No differences in fresh weight were found among crabs used in this experiment (one-way ANOVA, $F_{1,9}$=0.008, p=0.993). Also, no differences in CI were found among limpets used in the different treatments (one-way ANOVA, $F_{2,14}$=0.010, p=0.989).

### Statistical analyses

Differences in $O_2$-CR as well as enzyme activities among treatments were tested with separate one-way ANOVAs using treatments as a fixed factor (Zar 1999). An *a posteriori* Tukey HSD test corrected for unbalanced data was used to detect significant differences between pair of treatments (Hector et al. 2010). Prior to each ANOVA, the data were tested for normality (Shapiro–Wilks test) and homogeneity of variances (Cochran's test), and if necessary, were transformed to meet assumptions of ANOVA (Zar 1999). In the TBARs data set, we were not able to eliminate variance heterogeneity after logarithmic or arcsine transformation. Therefore, the nonparametric Kruskal–Wallis test was used to detect differences in TBARs content among treatments (Zar 1999).

## Results

### The effect of pea crabs on limpet metabolic rate

The $O_2$-CR of *Crepidula cachimilla* varied among the experimental treatments (one-way ANOVA: $F_{4,23}$=5.854, p=0.002; Fig. 2). The $O_2$-CR of long-term infested limpets (treatment T-1) varied between 0.065 and 0.176 mg $O_2$ $h^{-1}$ $g^{-1}$ (0.126±0.036, mean±SD), and this $O_2$-CR was ~2.5-fold higher than that of long-term uninfested limpets (treatment T-4, 0.051±0.032 mg $O_2$ $h^{-1}$ $g^{-1}$) and uninfested limpets+crabs (T-5, 0.046±0.025 mg $O_2$ $h^{-1}$ $g^{-1}$) (*a posteriori* Tukey test, p<0.01; Fig. 2). The presence of pea crabs did not in turn affect the $O_2$-CR of short-term infested limpets (T-2). The $O_2$-CR of these short-term infested limpets varied between 0.029 and 0.105 mg $O_2$ $h^{-1}$ $g^{-1}$ (0.055±0.030 mg $O_2$ $h^{-1}$ $g^{-1}$), and did not differ statistically from that of control limpets (T-4) (*a posteriori* Tukey test: p>0.05). The $O_2$-CR of short-term infested limpets (T-2) was considerably lower than that of long-term infested limpets (T-1) (*a posteriori* Tukey test: p<0.05). Lastly, the $O_2$-CR of short-term uninfested limpets (T-3, 0.083±0.040 mg $O_2$ $h^{-1}$ $g^{-1}$, range 0.038–0.136 mg $O_2$ $h^{-1}$ $g^{-1}$) was intermediate between that of long-term infested limpets and long-term uninfested (control) limpets (Fig. 2). However, differences between treatment T-3 and T-1 as well as T-3 and T-4 were not statistically significant (*a posteriori* Tukey test: p>0.05; Fig. 2).

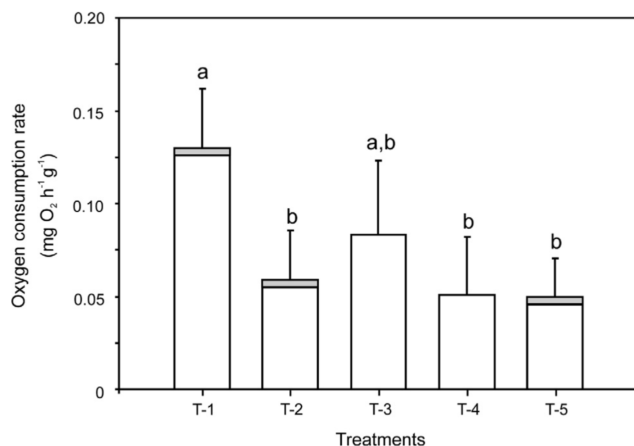


**Fig. 2.** Oxygen consumption rate ($O_2$-CR) of the slipper limpet host *Crepidula cachimilla* in five treatments (T-1, long-term infested limpet; T-2, short-term infested limpet; T-3, short-term uninfested limpet; T-4, long-term uninfested limpet; T-5, uninfested limpet+crab). Total height of bars in treatments T-1, T-2, and T-5 correspond to the $O_2$-CR of limpet+crab. After measurements in treatments T-1, T-2, and T-5, pea crabs were extracted from limpets and new $O_2$-CR readings of isolated pea crabs were conducted (see methods for further details). Then, the $O_2$-CR of isolated pea crabs (gray portion of bars) were subtracted from the initial measurements to obtain the $O_2$-CR of limpets (white portion of bars). Bars represent mean values and vertical lines indicate standard deviations. Different letters above the bars denote significantly different values at p<0.05 (*a posteriori* Tukey tests).

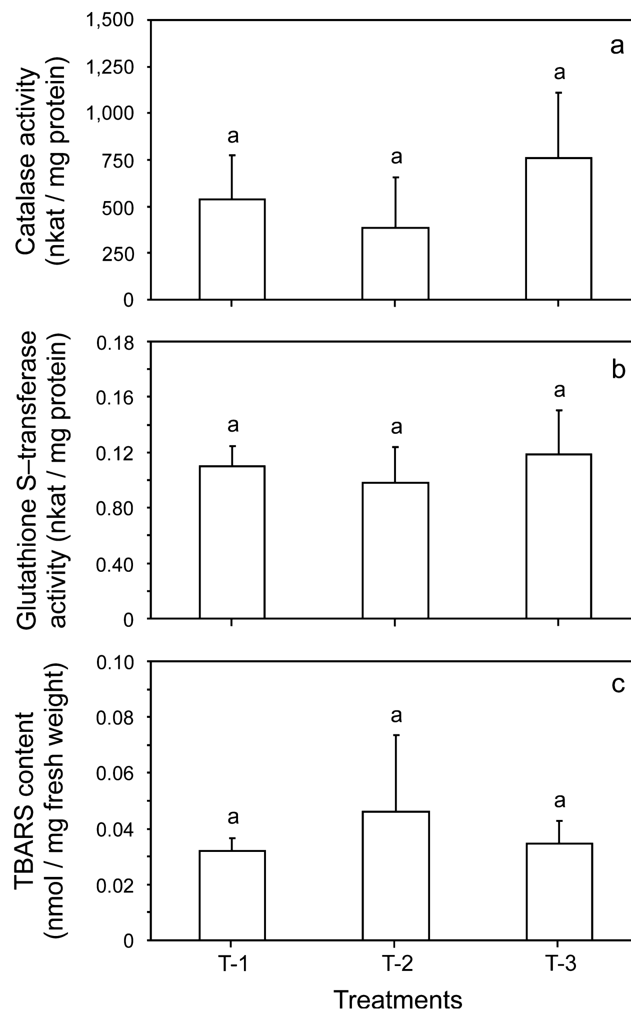


**Fig. 3.** Oxidative stress parameters in the gill of *Crepidula cachimilla* in three treatments (T-1, long-term infested limpet; T-2, short-term infested limpet; T-3, long-term uninfested limpet). **a**. Activity of the enzyme catalase (CAT). **b**. Activity of the enzyme glutathione S-transferase (GST). **c**. Content of thiobarbituric acid reactive substances (TBARs). Bars represent means, and vertical lines indicate standard deviations. Different letters above the bars denote significantly different values at p<0.05 (*a posteriori* Tukey tests).

The individual $O_2$-CR of limpets varied between 0.046 and 0.549 mg $O_2$ $h^{-1}$ $ind^{-1}$ (0.258±0.145, mean±SD) across all treatments. These values were more than one order of magnitude greater than the individual $O_2$-CR of isolated crabs, for which rates varied between 0.008 and 0.014 mg $O_2$ $h^{-1}$ $ind^{-1}$ ($O_2$-CR of crabs retrieved from T-1, 0.011±0.002 mg $O_2$ $h^{-1}$ $ind^{-1}$; T-2, 0.011±0.0014 mg $O_2$ $h^{-1}$ $ind^{-1}$; T-5, 0.012±0.0017 mg $O_2$ $h^{-1}$ $ind^{-1}$). These values of $O_2$-CR for isolated crabs represented 2.50%, 5.34%, and 6.32% of the limpet's $O_2$-CR in treatments T-1, T-2, and T-5, respectively (see Fig. 2). The $O_2$-CR of pea crabs was not different among treatments (one-way ANOVA, $F_{2,14}$=0.038, p=0.963).

### Limpet enzyme activities and lipid peroxidation

Female pea crabs did not measurably affect the oxidative status of limpets (Fig. 3). The activity of both CAT and GST was not statistically different among long-term infested, short-term infested, and uninfested limpets (one-way ANOVA: CAT, $F_{2,14}$=2.04, p=0.176; Fig. 3a; GST, $F_{2,14}$=0.852, p=0.453; Fig. 3b). Similarly, TBARs content did not differ statistically among treatments (Kruskal–Wallis test, H=0.295, p=0.863, with *n*=5, 6, and 6 limpets in T-1, T-2, and T-3, respectively; Fig. 3c).

## Discussion

Adult females of the slipper limpet *Crepidula cachimilla* harboring pea crabs (*Calyptraeotheres garthi*) for long time periods increase their metabolic rate compared with that of (control) long-term uninfested limpets. In contrast, short-term crab infesta-

tion did not affect the metabolic rate of host individuals, as demonstrated by the absence of statistical differences between the $O_2$-CR of short-term experimentally infested limpets and that of long-term uninfested limpets. The $O_2$-CR of limpets stripped of crabs shortly (24 h) before measurements was intermediate between that of long-term infested and uninfested limpets. Together, these results indicate that the pea crab *C. garthi* is a parasite, and suggest that the effect of pea crabs on the metabolic rate of limpets is reversible.

### The effect of pea crabs on limpet metabolic rate

There are several potential explanations for the observed increase in $O_2$-CR of long-term infested limpets. One is parasite-mediated shifts in the amount of energy that infested limpets partition among competing physiological activities such as maintenance, growth, and reproduction that, ultimately, lead to increased limpet metabolism (Scantlebury et al. 2007; Robar et al. 2011). For instance, females of *C. garthi* could inhibit host reproduction, as observed in a congeneric pea crab (Chaparro et al. 2001). Castrated limpets with a surplus of energy (due to the absence of reproductive activity) might increase resource allocation to somatic growth that, in turn, might lead to increased $O_2$-CR (see Hall et al. 2007; Lettini & Sukhdeo 2010). Nonetheless, no differences in condition index were found among limpets subjected to the different treatments in our experiments, suggesting that increases in energetically costly processes other than growth might be responsible for the increased metabolic rate in *Cr. cachimilla*.

Second, increased metabolism in hosts might occur if parasites cause injuries to host tissues or organs (e.g., gills), and if hosts augment resources devoted to repairing these damaged tissues (Delahay et al. 1995). Other pea crabs are known to damage gills or other host tissues (e.g., palps, mantle) while stealing food from hosts (e.g., Pearce 1966). We have observed females of *C. garthi* stealing food filtered by limpets during transportation from the gills to the mouth (Ocampo, unpubl. data). This kleptoparasitic behavior of *C. garthi* might damage the host food canal or gills, and any energy that limpets invest in repairing these structures might result in increased metabolic rate, as reported before in other host-parasite systems (Delahay et al. 1995). Lastly, slipper limpets could mount an energetically expensive immune response against pea crabs (Lochmiller & Deerenberg 2000; Giorgi et al. 2001; Klasing 2004), as do numerous other vertebrate and invertebrate hosts attacked by adult or larval parasitic forms (Belló et al. 2000; van de Crommenacker et al. 2012). In *Cr. cachimilla*, the epithelium of the pallial cavity might experience abrasion or small injuries given that *C. garthi* anchors itself inside the cavity using relatively thin but sharp pereopods (see Fig. 1). Such abrasions might increase the chances of bacterial infection, as demonstrated before in other parasites (e.g., the appendages of the copepod *Ostrincola koe* Tanaka 1961 cause skin irritation in the host clam *Meretrix meretrix* (Linnaeus 1758) that, in turn, increases susceptibility to microbial infections: Ho & Zheng 1994). The two mechanisms above (repairing tissues and immune response) might explain why short-term crab infestation does not affect the metabolic rate of host individuals; activation of tissue repair or immune responses might take longer than the 24-h duration of the short-term infestations. Future studies examining the effects of pea crabs on host tissues and putative damage experienced by infested limpets are necessary to reveal the mechanism underlying augmented metabolic rate in pea crab-infested limpets.

### Limpet enzyme activities and lipid peroxidation

In *Cr. cachimilla*, oxygen consumption rate increased in long-term infested compared with that of long-term uninfested limpets. Thus, we expected that this metabolic cost would affect the oxidative status of the infested limpets. However, the activity of two antioxidant enzymes (CAT and GST) and levels of lipid peroxidation did not vary between infested and uninfested limpets, irrespective of the length of time that limpets were experimentally infested. One possibility explaining our results is that oxidative stress does occur in *Cr. cachimilla*, but we failed to detect it because limpets used other antioxidant defenses (e.g., SOD, glutathione peroxidases, glutathione, α–tocopherol, or β–carotene) not evaluated in this work. For instance, Chambon et al. (2007) observed overexpression of the *Sod1* gene in the oyster *Crassostrea gigas* when infected by the polychaete worm *Polydora* sp., and a concomitant increase in oxygen concentration in the host's blood. New studies using other stress markers (e.g., SOD) might shed additional light on the oxidative response of *Cr. cachimilla* to increased $O_2$-CR.

Importantly, the link between metabolic activity and oxidant production is not straightforward. Although an excessive production of reactive oxygen species is expected when oxygen consumption increases, it is known that $O_2$ and $H_2O_2$ formation are not linearly related to activity of the regular

(i.e., cytochrome oxidase) electron transport chain (Brand 2000), and that mitochondria can sense environmental or body fluid $O_2$ tension and control respiratory activity in response (Guzy & Schumacker 2006). Furthermore, some organisms do exhibit alternative electron pathways (e.g., the alternative end oxidases (AOX) system; see Abele et al. 2007) for mitochondrial respiration so to reduce the production of reactive oxygen species. The gene coding for AOX has recently been identified in an oyster (McDonald & Vanlerberghe 2005), and it is likely present in all mollusks, including members of the family Calyptraeidae to which *Cr. cachimilla* belongs. Thus, if the AOX alternative pathway is activated in *Cr. cachimilla* when the regular electron transport rate saturates, increased $O_2$-CR would not necessarily translate into oxidative stress and the induction of antioxidant defenses CAT, GST, and lipid peroxidation levels could remain at basal levels, as observed in this study.

In summary, some studies have demonstrated that endosymbiotic invertebrates do affect the metabolism and increase the respiration rate of their hosts (e.g., Robles et al. 2001; Lettini & Sukhdeo 2010). Other studies have demonstrated oxidative stress that occurs concomitantly with augmented oxygen consumption rates (e.g., Pinho et al. 2005; da Rosa et al. 2008). However, there are only a handful of studies that have measured both $O_2$-CR and oxidative status in infested host individuals (e.g., Chambon et al. 2007). Our study has shown that, depending upon the infection period, pea crabs do increase host metabolism. However, this increase in metabolic rate does not appear to induce oxidative stress in hosts; increases in oxygen consumption rates are not necessarily coupled with cellular oxidative stress in infested host individuals (see Neves et al. 2000; Chambon et al. 2007). Several mechanisms might underlie the $O_2$-CR increase we observed, and further studies should be aimed at determining which of these are operating. Our results suggest that the effects of the parasite on host metabolism are reversible once the parasite is removed, although larger sample sizes may be necessary to detect any subtle differences in physiological responses among treatments.

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