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Energy metabolism and metabolic depression during exercise in *Callinectes sapidus*, the Atlantic blue crab: effects of the bacterial pathogen *Vibrio campbellii*

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SUMMARY

Callinectes sapidus **(Rathbun), the Atlantic blue crab, commonly harbors low to moderate amounts of bacteria in hemolymph and other tissues. These bacteria are typically dominated by *Vibrio* spp., which are known to cause mortality in the blue crab. The dose-dependent lethality of an isolate of *Vibrio campbellii* was determined in crabs; the mean 48 h LD_{50} (half-maximal lethal dose) was 6.2×10^5 colony forming units g^{-1} crab. Injection of a sublethal dose of *V. campbellii* into the hemolymph of the crab resulted in a rapid and large depression (30–42%) of metabolic rate, which persisted for 24 h. Because gills are an organ of immune function as well as respiration, we were interested in how bacteria injected into the crab would affect the energetic costs associated with walking. Overall metabolism (aerobic and anaerobic) more than doubled in crabs walking for 30 min at 8 m min^{-1}. The metabolic depression resulting from bacterial injection persisted throughout the exercise period and patterns of phosphagen and adenylate consumption within walking leg muscle were not affected by treatment. The ability of crabs to supply required energy for walking is largely unaffected by exposure to *Vibrio*; however, *Vibrio*-injected crabs are less aerobic while doing so. This depressed metabolic condition in response to bacteria, present during moderate activity, could be a passive result of mounting an immune response or may indicate an actively regulated metabolic depression. A compromised metabolism can affect the performance of daily activities, such as feeding and predator avoidance or affect the ability to cope with environmental stressors, such as hypoxia.**

Key words: *Callinectes sapidus*, blue crab, LD_{50}, oxygen uptake, lactate, metabolic depression, immune defense, *Vibrio*.

INTRODUCTION

The Atlantic blue crab, *Callinectes sapidus* (Rathbun), an important species for commercial and recreational fishing (Whitaker et al., 1998), lives in estuarine and other coastal environments along the Atlantic and Gulf coasts of North America. In these habitats, seasonal and diurnal fluctuations of salinity, temperature, oxygen and pH (deFur et al., 1990; Mikulski et al., 2000) can profoundly impact physiological processes. Such changes in environmental conditions not only alter fundamental processes of osmoregulation, respiration, circulation, digestion and acid–base balance (for a review, see Towle and Burnett, 2006), but also immune defenses (Newman and Feng, 1982; Le Moullac and Haffner, 2000; Holman et al., 2004; Burgents et al., 2005a; Macey et al., 2008). Interestingly, new evidence suggests that mounting an immune response against bacterial infection interferes with circulation, and consequently respiration, within crustaceans (Burnett et al., 2006; Scholnick et al., 2006).

Bacteria of the genus *Vibrio* occur naturally in marine environments and are frequently associated with the exoskeleton and hemolymph of blue crabs (Colwell et al., 1975; Tubiash et al., 1975; Sizemore and Davis, 1985; Welsh and Sizemore, 1985). *Vibrio* spp. are characterized as opportunistic crustacean pathogens and can cause mortality in the blue crab (Krantz et al., 1969; Johnson, 1976; Welsh and Sizemore, 1985). Experimentally introduced bacteria or other foreign particles are rapidly cleared (within 1 h) from crustacean hemolymph (McCumber and Clem, 1977; Merrill et al., 1979; White and Ratcliffe, 1982; Adams, 1991; Martin et al., 1993; van de Braak et al., 2002; Holman et al., 2004). The gills are particularly important sites for accumulation and subsequent removal of these bacteria or particles (Smith and Ratcliffe, 1980b; White and Ratcliffe, 1982; Martin et al., 1993; Martin et al., 2000).

Adult blue crabs, like many portunid crabs, are most noted for their proficient swimming capabilities but they are also capable of walking along the bottom, most often laterally, using three paired walking appendages (Spirito, 1972). Tagging studies have shown that blue crabs typically move 50–900 m within estuaries on a daily basis, but may migrate seasonally over larger distances of up to 800 km, using a combination of swimming high in the water column on flood tides and walking along the bottom on ebb tides (Hines, 2007). However, there are currently no data available on the energetics of walking in the blue crab, as studies of exercise physiology in blue crabs have been largely focused on swimming (Booth et al., 1982; Booth and McMahon, 1985).

Exercise by either swimming or walking in decapod crustaceans is, as in most animals, associated with an increase in the metabolic demand for oxygen (McMahon, 1981). There is anywhere from a two- to a sevenfold elevation of oxygen uptake during increased activity, made possible by increases in ventilation rate, heart rate and cardiac output (Herreid et al., 1979; McMahon et al., 1979; Wood and Randall, 1981; Booth et al., 1982; Taylor, 1982; Full and Herreid, 1984; Houlihan and Innes, 1984; Houlihan et al., 1984; Houlihan et al., 1985; Hamilton and Houlihan, 1992). Elevated oxygen uptake persists for some time after activity is completed. This is characterized as excess post-exercise oxygen consumption (EPOC), which is used to recharge depleted energy stores and to dispose of anaerobic end products generated during activity

(McMahon, 1981; Gaessar and Brooks, 1984; Gleeson and Hancock, 2001; Hancock and Gleeson, 2002). Highly aerobic crustaceans, such as the blue crab, typically have a small oxygen deficit recovery (e.g. Booth et al., 1982) and oxygen uptake often returns to pre-activity levels within 20–45 min (Herreid et al., 1979; Booth et al., 1982; Full and Herreid, 1983; Houlihan and Innes, 1984; Hamilton and Houlihan, 1992). However, in organisms more reliant on anaerobic pathways to fuel energy demands during activity, this recovery may take as long as 5–24 h (McDonald et al., 1979; McMahon et al., 1979; Wood and Randall, 1981). Of course, this also partially depends on the duration and intensity of the activity performed.

It is perhaps surprising that aerobic metabolism in shrimp (Scholnick et al., 2006) and in blue crabs (Burnett et al., 2006) decreases profoundly following injection of a sublethal dose of bacteria. This metabolic depression is especially interesting since the gills are implicated in the immune response (Smith and Ratcliffe, 1980a; White et al., 1985; Martin et al., 2000) during which time their respiratory function declines (Burnett et al., 2006). The decline is rapid and, in the case of the shrimp, it persists for 24 h. We were interested in investigating this phenomenon further in the blue crab and determining whether bacterial injection impaired the response of the blue crab to exercise.

MATERIALS AND METHODS

Animal collection and maintenance

Adult male blue crabs, *Callinectes sapidus* (60–140 g), were collected locally in Charleston, SC, USA and held at the Hollings Marine Laboratory in well-aerated, recirculating seawater (30‰; 20–22°C; pH 7.5–8.5) on a 12 h:12 h dark:light cycle for a minimum of 3 days but no longer than 2 weeks before use in experiments. Crabs were fed daily with squid that were previously frozen, but food was withheld at least 1 day prior to and during each experiment. All experiments were performed in filtered seawater (30‰ salinity).

Animal preparation and assessment

Crabs were prepared for injection as described by Holman et al. (Holman et al., 2004). At least 24 h before injecting bacteria (referred to as a bacterial challenge), a 1 mm hole was drilled through the carapace directly over the heart as a port for injection of bacteria or saline into the ventricle. Injection of bacteria directly into the ventricle ensured rapid and even distribution of the injected bacteria by the circulatory system as described by Macey et al. (Macey et al., 2008). Two similar holes were drilled over the pericardial sinus adjacent to the heart for sampling hemolymph from the pericardial space. A thin layer of latex rubber was secured over the drilled holes with cyanoacrylate glue and acted as a diaphragm to allow for injection or sampling of hemolymph with a needle. Each crab was placed individually in a small container with 1.5 l well-aerated 30‰ seawater and a thin layer of gravel.

To assess crabs for a pre-existing systemic infection with culturable bacteria, 150 µl of hemolymph was sampled from the pericardial sinus, diluted 1:10 in sterile 10 mmol l^{-1} Hepes-buffered 2.5% NaCl (hereafter referred to as saline), suspended in marine agar and overlaid onto a sterile tryptic soy agar (TSA; Difco, Becton Dickenson and Co., Sparks, MD, USA) microbial culture plate supplemented with 2% NaCl. These plates were incubated for 24 h at 25°C and then examined for colony forming units (CFU) of bacteria. Only those crabs with no culturable bacteria present in the hemolymph, as detected by this assay, were used for experimentation.

Bacterial preparation

The bacterial pathogen used was *V. campbellii* 90-69B3, stably transfected with the *Vibrio*-derived plasmid EVS146 that expresses green fluorescent protein and resistance to antibiotics kanamycin and chloramphenicol (Dr E. Stabb, University of Georgia). The parental strain of *V. campbellii* 90-69B3 was isolated from diseased shrimp *Litopenaeus vannamei* by D. Lightner and L. Mahone (University of Arizona). *V. campbellii* is an opportunistic pathogen, and its pathogenicity in *L. vannamei* is comparable to that of other marine *Vibrio* spp. that are commonly isolated from marine waters (Mikulski et al., 2000). *V. campbellii* was selected for the present study because its distribution, inactivation and elimination from tissues of shrimp and crabs have been extensively documented (Holman et al., 2004; Burgents et al., 2005b). Stability of the plasmid and growth characteristics of the transfected strain have been described previously (Burgents et al., 2005b).

One day prior to injection, *V. campbellii* was streaked from a frozen glycerol working stock onto a TSA plate containing 2.5% NaCl, 100 µg ml^{-1} kanamycin A (Sigma-Aldrich, St Louis, MO, USA) and 5 µg ml^{-1} chloramphenicol (Sigma-Aldrich) and incubated at 25°C for 24 h. Bacteria from this plate were then transferred to a tube containing sterile 10 mmol l^{-1} saline using a sterile inoculating loop and the bacterial concentration was adjusted to an optical density of 0.1±0.005 at 540 nm, which equals a bacterial concentration of 10^8 CFU ml^{-1} (Mikulski et al., 2000; Burgents et al., 2005b). The prepared bacterial suspension was adjusted with sterile saline to obtain the desired concentration for injection. Injection volumes of saline or saline containing *Vibrio* never exceeded 300 µl.

Determination of bacterial LD_{50}

Lethality of *V. campbellii* 90-69B3 in the blue crab was assessed by performing three 48-h LD_{50} bacterial challenges. In each replicate experiment, crabs (60–125 g; N=7 for each dose) were injected with saline (control) or one of three doses (2.5×10^4, 2.5×10^5 or 2.5×10^6 CFU g^{-1} crab) of *V. campbellii*. The inoculum of bacteria for each injection was prepared as described above to obtain a concentration of 10^8 CFU ml^{-1} saline. To prepare the lowest dose, this bacterial suspension was diluted 1:3 with sterile saline to obtain a concentration of 2.5×10^7 CFU ml^{-1}. This concentration, when injected as 1 µl g^{-1} crab, delivered a dose of 2.5×10^4 *Vibrio* g^{-1} crab. Animals receiving the mid-range dosage were injected with 2.5 µl g^{-1} crab of the original bacterial suspension (10^8 CFU ml^{-1} saline) which delivered the desired dose of 2.5×10^5 CFU g^{-1} crab. To prepare the highest bacterial challenge dose, 10 ml of the original bacterial suspension of 10^8 CFU ml^{-1} saline was pelleted by centrifugation (2000 ***g*** for 5 min), the supernatant discarded, and the pellet resuspended in 1 ml of saline to obtain a concentration of 10^9 CFU ml^{-1}. Each animal was injected with 2.5 µl g^{-1} crab of the concentrated bacteria to deliver a final dose of 2.5×10^6 CFU g^{-1} crab. Control animals received an injection of 1 µl g^{-1} crab of sterile saline.

In each replicate trial, crabs were injected directly into the ventricle with the appropriate dose of saline containing *Vibrio* or saline (control) within their individual containers ensuring that the gill chambers stayed fully submerged while doing so. The water within each container was changed every 24 h during the experiment. Mortality was recorded at 0, 2, 4, 8, 12, 24 and 48 h following bacterial challenge.

Bacterial doses for experiments

Based on our determination of an LD_{50} dose of *V. campbellii* (methods described above and reported in Results), we used a

sublethal dose of 2.5×10^4 CFU g^{-1} crab for subsequent experiments. This is equivalent to a circulating dose of 1×10^5 CFU ml^{-1} hemolymph, assuming a hemolymph volume of 25 ml 100 g^{-1} body mass (Gleeson and Zubkoff, 1977).

Oxygen uptake and hemolymph lactate in resting crabs

Oxygen uptake following the injection of a sub-lethal dose of *V. campbellii* was measured at 25°C using a flow-through respirometry system. The P_{O_2} of seawater entering and leaving a sealed 1.8 l circular, plexiglass respirometry chamber (17 cm wide, 8 cm high) lined with sterile gravel was measured every 10 s using an O_2 electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) and a Sable System (Las Vegas, NV, USA) data acquisition system. Seawater entering the chamber was gassed with 30% O_2 (31 kPa) to ensure that the partial pressure within the chamber did not drop below 80% air saturation (16.5 kPa O_2) when a crab was present in the chamber. Flow rates through the chamber usually ranged between 80 and 150 ml min^{-1}.

Typically, a crab was placed in the respiratory chamber, then the chamber was sealed, covered and the animal left undisturbed for 2 h. After this time, the chamber was opened and the crab was injected with saline (control) or saline containing *V. campbellii* (2.5×10^4 CFU g^{-1} crab). The chamber was then resealed and O_2 uptake was measured continuously for 4 h. Afterwards, the crab was removed from the respiratory chamber and held overnight in an individual container of well-aerated 30‰ seawater at 25°C. Approximately 22 h after injection, the crab was returned to the sealed respiratory chamber and left undisturbed for 2 h. A final measurement of O_2 uptake over 30 min was recorded.

In a separate group of crabs, changes in anaerobic metabolism were assessed by measuring hemolymph lactate levels over time after injection of saline or *V. campbellii*. Hemolymph (30 μl) was sampled from the pericardial space of each crab at 30 min, 2 h, 4 h and 24 h following injection and immediately deproteinized in ice-cold 12% perchloric acid (1:10 dilution). The samples were then centrifuged and lactate concentrations measured enzymatically using a NADH-linked colorimetric assay (Sigma Technical Bulletin No 862-UV; lactate dehydrogenase, Sigma #L2500) and modified according to Graham et al. (Graham et al., 1983).

Exercise protocol

Walking was initiated and sustained on a variable speed treadmill equipped with a flow-through respirometry system. The treadmill is enclosed in a 4.3 l clear, watertight plexiglass chamber and powered with an external DC motor. The crabs were able to move freely about in the 18 cm×17 cm×7 cm high compartment above the tread. In each experiment, crabs were injected with saline (control) or saline containing *V. campbellii*, placed within the treadmill chamber, and allowed to rest for 1 h. Animals then walked on the treadmill at a speed of 8 m min^{-1} for 30 min. After this activity was completed, each crab was allowed to recover for 3 h.

Three different sets of experiments employed this apparatus and activity regime. In the first set of experiments, O_2 uptake was measured in 14 crabs (N=7 each for saline-injected and *Vibrio*-injected crabs) using flow-through respirometry. In the second, hemolymph was sampled for lactate analysis from a different set of 18 crabs (N=9 for each injection treatment) at designated timepoints during the activity regime. In the final set of experiments, a total of 84 crabs were sampled at designated times to quantify whole body lactate and muscle metabolites.

Oxygen uptake and hemolymph lactate during and after exercise

O_2 uptake was measured at 25°C in the treadmill respirometer as described above. Flow rates through the chamber ranged between 80 and 200 ml min^{-1}. Instantaneous measurements of O_2 uptake can be obtained by the difference in O_2 pressures in the water flowing into and out of the chamber at a known water flow rate when the rate of O_2 uptake is constant, as observed under resting conditions. However, when the oxygen uptake rate of the animal changes to a new steady-state level, there is a delay in the establishment of a new steady-state P_{O_2} of the water exiting the respirometer. The approach to a new steady-state level is an exponential function of time. This delay or 'washout time' has been described by Frappell et al. (Frappell et al., 1989) and was accounted for in the present study by applying the Z transformation (Bartholomew et al., 1981). In order to minimize any washout effects associated with the transition from resting O_2 uptake to that which occurs during walking and the early stages of recovery, water flow through the respirometer was increased during activity. Increasing the flow rate during the activity period decreases the washout time and insures that the O_2 pressure within the chamber stays reasonably constant and does not drop below 80% air saturation. In fact, P_{O_2} of the water exiting the treadmill chamber during walking changed very little from resting values as a result of increasing the water flow rate.

Each crab was placed in the treadmill chamber and left undisturbed for 2 h, during which time well-oxygenated seawater passed through the chamber. The lid of the chamber was then removed and without removing the crab from the water, saline (control) or saline containing *V. campbellii* was injected directly into the ventricle though the injection port. The chamber was resealed and the animal was allowed to rest in the treadmill chamber for 1 h. Each crab then walked on the treadmill for 30 min as described above. Subsequently, the treadmill was stopped and O_2 uptake for each animal was recorded during a 3 h recovery period. Excess post-exercise oxygen consumption (EPOC) was calculated by integrating the area described by the elevation of O_2 uptake above the base-line value from 0 to 80 min after exercise, by which time O_2 uptake had returned to base-line levels.

Hemolymph lactate concentration was measured on a separate group of crabs injected with saline (control) or saline containing *V. campbellii* and exercised according to the same regimen. Each crab was injected with saline (control) or saline containing *V. campbellii* into the ventricle while remaining submerged in its individual container. Hemolymph (30 μl) was sampled from the pericardial sinus after 1 h at rest in the treadmill chamber, immediately after 30 min of walking, and at 1, 2 and 3 h during the recovery period. Hemolymph samples were immediate deproteinized and lactate was measured as described above.

Whole body and muscle metabolites during and after exercise

An additional 84 crabs were injected, exercised and rested following the same regimen described above. To measure metabolites in the whole crab and separately in muscle tissues at different times before, during and after the exercise period, crabs were removed rapidly from the apparatus at the appropriate time and plunged into liquid nitrogen to freeze all tissues. Crabs were frozen at the following times: 1 h after injection and prior to walking, immediately after 30 min of walking on the treadmill, and 5, 30, 60, 120 and 180 min after walking. Frozen crabs were removed from the liquid nitrogen and cut (anterior to posterior) into two equal halves and stored at –80°C until analysis. Whole body lactate and succinate were

measured in one half of the crab. Lactate, succinate, phosphagens and adenylates were measured in the leg muscle (described below).

To measure whole body lactate and succinate one half of each crab was cryogenically homogenized using a Teflon disk mill (W. P. Law, Johns Island, SC, USA). The intact frozen crab tissue was broken up into smaller pieces with a sledgehammer and placed in a Teflon disk mill that was pre-cooled overnight in a XLC1211 liquid N_2 vapor-phase freezer (Chart Industries-MVE, Marietta, GA, USA) and powdered using a TS-250X Teflon Disk Mill Shaker (Siebtechnik GMBH, Mülheim an der Ruhr, Germany). The powdering process took less than 2 min and transformed the frozen tissue into a uniformly homogeneous particulate powder (Pugh et al., 2007). The powder was transferred to a small container for storage at –80°C until analysis. A known mass (~0.4 g) was transferred to a tube containing three parts ice-cold 0.9 mmol l^{-1} perchloric acid ($HClO_4$) and homogenized while on ice for 30 s at 30,000 r.p.m. using a Polytron PT 2100 homogenizer system (Westbury, NY, USA). Samples were transferred to 2 ml microcentrifuge tubes and centrifuged for 10 min at 11,000 ***g*** at 4°C. The supernatant was removed from the microcentrifuge tube and the remaining pellet was washed with 0.2 mmol l^{-1} $HClO_4$ using one-third the volume of the initial extraction, and centrifuged as stated above. The pooled supernatant from the two previous steps was neutralized with a volume of 3.75 mmol l^{-1} potassium carbonate (K_2CO_3) equal to 10% of the total amount of perchloric acid used in both extraction steps. The samples were shaken vigorously and centrifuged a final time for 10 min at 11,000 ***g*** at 4°C and the resulting supernatants were analyzed for lactate and succinate. Lactate was assayed as described above for hemolymph. Succinate concentrations were measured in a subset of samples using a commercially available NADH-linked colorimetric assay kit (R-Biopharm, Marshall, MI, USA).

Leg muscles, the abductor and adductor carpopoditus (Cochran, 1935), taken from the merus of the second walking leg, were removed frozen from the exoskeleton of the second crab half and powdered under liquid nitrogen in a pre-cooled mortar and pestle. A known mass (~0.4 g) was transferred to a tube containing three parts ice-cold 0.9 mmol l^{-1} perchloric acid ($HClO_4$) and homogenized as described above. Arginine and arginine phosphate were analyzed as described by Bergmeyer (Bergmeyer, 1985a), with modifications described by Morris and Adamczewska (Morris and Adamczewska, 2002) and Morris et al. (Morris et al., 2005). Briefly, free arginine was first measured by the change in absorbance at 340 nm in the NADH-linked conversion of arginine and pyruvate to octopine by octopine dehydrogenase. In order to measure arginine phosphate, 100 µl of 1 mol l^{-1} HCl was added to 100 µl sample and incubated on a heat block at 100°C for 2 min to hydrolyze arginine phosphate into arginine and phosphate. The hydrolyzed subsample was immediately cooled on ice and neutralized with an equal part of 1 mol l^{-1} NaOH. The assay was repeated and the previously measured amount of arginine was subtracted from the total arginine concentration to obtain the amount of arginine phosphate present in the sample. From this, the arginine charge (Schulte et al., 1992) of the muscle can be calculated as:

$$\mathrm{ArgEC} = \frac{[\mathrm{PArg}]}{[\mathrm{PArg}] + [\mathrm{Arg}]} \,. \quad (1)$$

Muscle lactate and succinate were measured as described above. ATP, ADP and AMP concentrations in the muscle were analyzed with NADH-linked assay methods as described by Bergmeyer (Bergmeyer, 1985b). The oxidation of NADH, measured by the change in absorbance at 340 nm, is proportional to the amount of adenylate present (Morris et al., 2005). From these data, the adenylate energy charge (AEC) (Atkinson, 1968) can be calculated as follows, which describes the overall energetic status of the muscle:

$$\mathrm{AEC} = \frac{[\mathrm{ATP}] + \frac{1}{2}[\mathrm{ADP}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]} \,. \quad (2)$$

Data analysis

Statistical analyses were performed using SigmaStat 3.0. All data are given as means ± s.e.m. Significance level is α=0.05 for all tests.

The effect of bacterial challenge dose on the percentage survival of blue crabs over 96 h was analyzed by two-way ANOVA. *Post-hoc* comparisons between crabs challenged with bacteria and crabs injected with saline (control group) were performed using the Holm–Sidak (HS) method. 48 h LD_{50} values and confidence intervals for each replicate experiment were calculated by the trimmed Spearman–Karber method (Hamilton et al., 1977). This particular method is a non-parametric statistical procedure that estimates the trimmed mean LD_{50} and associated 95% confidence intervals from a distribution of the $\log_{10}$ of percentage mortality over the range of exposure concentrations after a fixed exposure time.

For experiments with resting crabs, oxygen uptake and hemolymph lactate data were analyzed using one-way or two-way repeated measures ANOVA (RM-ANOVA) to allow comparisons between treatment groups (saline *vs Vibrio*) and with time. The oxygen uptake data were not normally distributed so the data were ranked and the ANOVA analysis performed on the ranked data. Hemolymph lactate data were transformed using the square root to achieve normality. *Post-hoc* comparisons between treatments (saline *vs Vibrio*) at individual timepoints were performed using the HS method.

For experiments with exercised crabs, O_2 uptake data were compared between treatments (saline *vs Vibrio*) and over time using repeated measures (RM) ANOVA. Data were $\log_{10}$ transformed to achieve normality. If a statistically significant difference between treatments was found, *post-hoc* comparisons of individual timepoints between treatments were performed using the HS method. A significant difference in time within treatment resulted in a *post-hoc* comparison of resting oxygen uptake values with values measured during and after activity within each treatment using the HS method.

All lactate, succinate and muscle metabolite data were analyzed using two-way ANOVA to compare between treatments (saline *vs Vibrio*) and between timepoints within each treatment. Values for whole body lactate and hemolymph lactate were $\log_{10}$ transformed to achieve normality. *Post-hoc* comparisons using the HS method were employed when a significant difference between treatments was found. When a significant difference was indicated between timepoints, *post-hoc* analysis using the HS method was used to compare pre-activity levels with values measured during recovery within each treatment.

RESULTS

Bacterial LD_{50}

V. campbellii was lethal to *C. sapidus* in a dose-dependent manner (Fig. 1). There was a significant effect of injection dose on the survival of blue crabs over time (two-way ANOVA; P<0.001). Only 1 out of 21 saline-injected crabs died during experimental trials. All mortality occurred within 48 h following injections. No significant

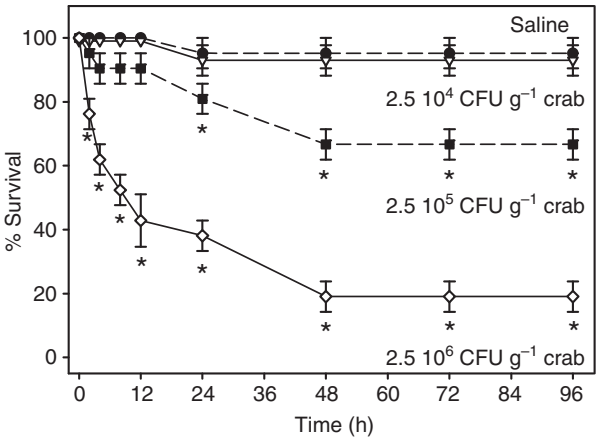

Fig. 1. Percentage survival of *C. sapidus* over time after injection of *V. campbellii* or saline (control). Values shown are the mean ± s.e.m. of three replicate trials (N=7 per trial) for each treatment. There was a significant effect of injection treatment on the survival of blue crabs over 96 h (two-way ANOVA; P<0.001). *A significant difference in percentage survival between treatments at individual timepoints (HS; P<0.02).

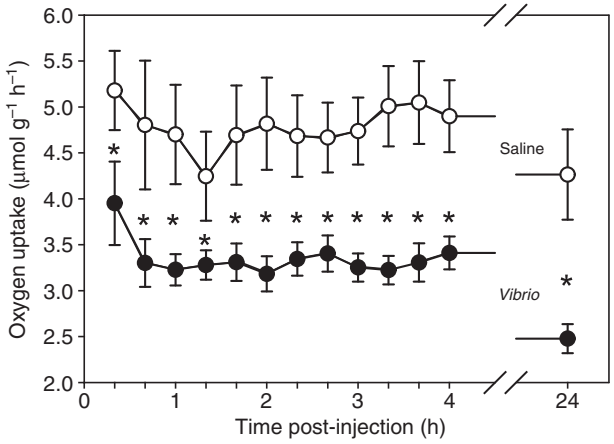

Fig. 2. Oxygen uptake in *C. sapidus* following injection of *V. campbellii* (filled circles) or saline (control; open circles). Values represent averages over 20-min intervals for seven animals (± s.e.m.). There was a significant effect of injection of *V. campbellii* on oxygen uptake over 24 h (two-way RM ANOVA; P=0.0012). *A significant difference in oxygen uptake between treatments at individual timepoints (HS; P<0.03).

difference was found in percentage survival between crabs receiving saline injections and those receiving *Vibrio* injections of 2.5×10^4 CFU g^{-1} crab (Fig. 1). However, percentage survival was significantly lower in crabs receiving an injection of 2.5×10^5 CFU g^{-1} crab after 24 h compared with control crabs (HS; P<0.02). Crabs injected with 2.5×10^6 CFU g^{-1} crab exhibited a significantly lower percentage survival after only 2 h compared with crabs receiving saline injections (HS; P<0.001). 48 h LD_{50} values were analyzed using the trimmed Spearman–Karber method (Hamilton et al., 1977) and ranged from 5.1×10^5 CFU g^{-1} crab to 7.9×10^5 CFU g^{-1} crab with an average value of 6.2×10^5 CFU g^{-1} crab. The 95% confidence interval for these data ranged from 1.2×10^5 CFU g^{-1} crab to 2.9×10^6 CFU g^{-1} crab across all three replicates.

Oxygen uptake and hemolymph lactate in resting crabs

Prior to injection, oxygen uptake rates in saline-injected crabs (3.52±0.46 μmol g^{-1} h^{-1}) and *Vibrio*-injected crabs (2.89±0.11 μmol g^{-1} h^{-1}) were not significantly different from one another (one-way ANOVA; P=0.233). Injection of *V. campbellii* caused a rapid and sustained reduction in oxygen uptake over a 24 h period (Fig. 2; two-way RM ANOVA on ranked data; P=0.012). A 24% reduction in oxygen uptake occurred within the first 20 min after injection compared with saline-injected crabs (HS; P<0.05). Oxygen uptake in *Vibrio*-injected crabs was reduced by 30% after 4 h and was further reduced to 42% after 24 h compared with oxygen uptake measured in saline-injected crabs (HS; P<0.05).

Although hemolymph lactate levels were generally low (<1 mmol l^{-1}), there was a significant effect of injection treatment on hemolymph lactate over a 24 h period (two-way RM ANOVA; P=0.004). Accumulation of lactate in the hemolymph was significantly greater in *Vibrio*-injected crabs compared with saline-injected crabs 2 h following injection; this difference in hemolymph lactate persisted for 24 h (Fig. 3; HS; P<0.03). Hemolymph lactate levels in saline-injected crabs were different only between the 0.5 h and the 24 h timepoints (Fig. 3; one-way RM ANOVA with HS; P<0.02) whereas levels in *Vibrio*-injected crabs did not significantly change with time.

Walking activity on the treadmill

Blue crabs are capable of walking on a treadmill at a moderate pace of 8 m min^{-1} for 30 min. Blue crabs mainly walked laterally, but occasionally walked backwards, using three pairs of walking legs while the chelipeds and swimming legs were mainly used for stabilization. Animals also occasionally rotated 180° during activity, a behavior commonly observed in blue crabs (Spirito, 1972). Crabs subjected to either treatment (saline or *Vibrio*) were able to perform this level of activity, so a direct comparison of measurements between treatments could be made.

Oxygen uptake and hemolymph lactate during and after exercise

Oxygen uptake significantly changed with activity in both saline-injected crabs and *Vibrio*-injected crabs (one-way RM ANOVA; P<0.001 for both treatments). Oxygen uptake increased by 2.1–2.5 times resting levels during activity in both treatment groups (HS; P<0.03 for both treatments) and reached steady state within the first 10 min (Fig. 4). Animals quickly recovered from activity and oxygen uptake levels remained elevated only during the first 40 min of recovery from walking (HS; P<0.01 for both treatments).

Significant differences in oxygen uptake were found between treatment groups (two way RM ANOVA; P=0.012). Oxygen uptake in saline-injected crabs at rest before exercise (4.59±0.37 μmol g^{-1} h^{-1}) was similar to that found in *Vibrio*-injected crabs (3.65±0.4 μmol g^{-1} h^{-1}). However, oxygen uptake was significantly reduced during walking in *Vibrio*-injected crabs (7.74±0.88 μmol g^{-1} h^{-1}) compared with saline-injected crabs (11.52±0.89 μmol g^{-1} h^{-1}; HS; P<0.03). *Vibrio*-injected crabs continued to consume significantly lower amounts of oxygen during 3 h of recovery from walking (Fig. 4; HS; P<0.03).

Hemolymph lactate levels increased with activity (one-way ANOVA; P<0.02 for both treatments) and were significantly different between saline-injected and *Vibrio*-injected crabs (Fig. 5A; two-way ANOVA; P<0.001). Hemolymph lactate was only slightly elevated in saline-injected crabs after activity (1.00±0.19 mmol l^{-1}; HS; P=0.004) and quickly returned to pre-activity levels within 1 h during recovery (Fig. 5A). Hemolymph lactate in *Vibrio*-injected

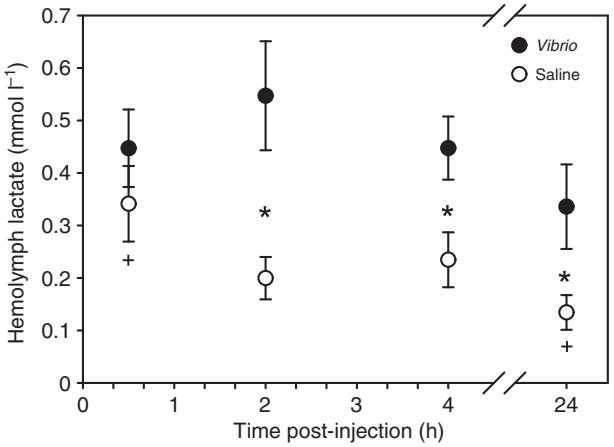

Fig. 3. Concentration of lactate in the hemolymph of *C. sapidus* following injection of *V. campbellii* (filled circles) or saline (control; open circles). Values are means ± s.e.m. for nine crabs. There was a significant effect of injection treatment on circulating hemolymph lactate levels over 24 h (two-way RM ANOVA; P=0.004). *A significant difference in hemolymph lactate between treatments at individual timepoints (HS; P<0.03). A plus sign indicates a significant difference in hemolymph lactate between these two timepoints within the saline-injected group (one-way RM ANOVA; HS P=0.004).

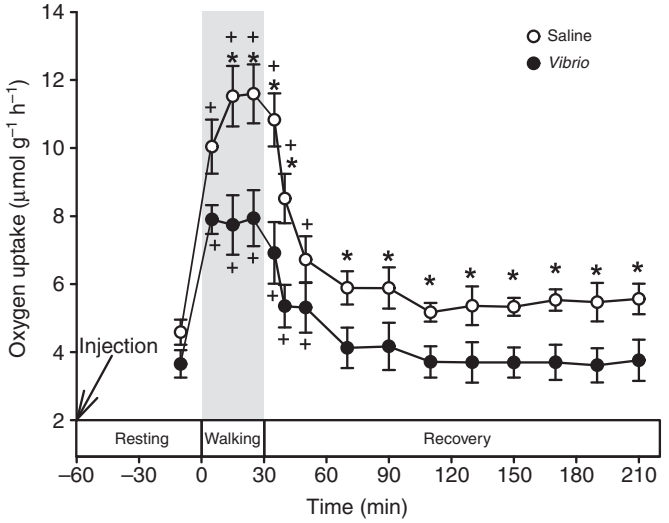

Fig. 4. Oxygen uptake (μmol g^{-1} h^{-1}) in *C. sapidus* injected with *V. campbellii* (closed circles) or saline (control; open circles). The crabs were rested for 1 h after injection and then measurements were taken during 30 min of walking at 8 m min^{-1}, and during a 3 h recovery period. Values are means ± s.e.m. N=7 for each treatment group. Oxygen uptake increased significantly over time in both saline-injected crabs and *Vibrio*-injected crabs (one-way RM ANOVA; P<0.001 for both treatments). There was also a significant difference in oxygen uptake between treatments during activity and recovery (two-way RM ANOVA; P=0.012). A plus sign indicates a significant increase in oxygen uptake compared with resting values within each treatment (HS; P<0.01). *A significant difference in oxygen uptake between treatments at individual timepoints (HS; P=0.012).

crabs increased considerably during activity (HS; P=0.001), and was significantly higher (3.63±0.67 mmol l^{-1}) immediately after activity (HS; P<0.001); this elevation in hemolymph lactate above control animals persisted during 2 h of recovery (Fig. 5A).

Whole body and muscle metabolites during and after exercise

Whole body lactate (Fig. 5B) changed over time in both treatment groups (one-way ANOVA; P=0.002 for both treatments). Resting whole body lactate averaged 7.12±0.66 μmol g^{-1} in *Vibrio*-injected crabs and 5.86±0.46 μmol g^{-1} in saline-injected crabs (Fig. 5B). Whole body lactate significantly increased ~1.8-fold during activity to 11.77±1.88 μmol g^{-1} in *Vibrio*-injected crabs and 10.53±1.15 μmol g^{-1} in saline-injected crabs (HS; P<0.01 for both treatments). Elimination of whole body lactate was slow, with values reaching pre-activity levels within 2 h of recovery (150 min after the onset of walking; Fig. 5B). Although whole body lactate in saline-injected crabs declined immediately after exercise, whole body lactate in *Vibrio*-injected crabs increased to 12.58±1.00 μmol g^{-1} at 30 min recovery. As a result, there was significantly more whole body lactate present in *Vibrio*-injected crabs at 30 min recovery than in saline-injected crabs (Fig. 5B; two-way ANOVA with HS, P=0.005).

Leg muscle (from the merus of the second walking leg) lactate significantly increased with activity overall (one-way ANOVA; P<0.01 for both treatments) and there were significant differences between treatment groups (two-way ANOVA; P=0.007). *Vibrio*-injected crabs had significantly more muscle lactate (6.29±0.29 μmol g^{-1}) that saline-injected crabs (3.48±0.61 μmol g^{-1}) at rest (Fig. 5C; HS; P=0.007). Muscle lactate increased significantly during activity to 10.53±1.16 μmol g^{-1} in *Vibrio*-injected crabs and 7.65±1.08 μmol g^{-1} in saline-injected crabs; the difference between injection treatments persisted through 30 min of recovery (Fig. 5C; HS; P<0.03). Similarly to that observed in the whole body, muscle lactate levels dropped more rapidly in saline-injected crabs, resulting in significantly more muscle lactate present in *Vibrio*-injected crabs (9.47±1.06 μmol g^{-1}) at 30 min recovery than in saline-injected crabs (6.31±0.35 μmol g^{-1}) (Fig. 5C; HS; P=0.017).

Selected samples of whole body and leg muscle tissues were analyzed for succinate, an alternative anaerobic end product generated by many invertebrates (Livingstone, 1991). Succinate concentrations in the whole body and leg muscle were very similar and ranged from 0.6 to 1 μmol g^{-1} in both tissues (Table 1). Succinate levels did not significantly change with activity in either tissue type (two-way ANOVA; P>0.05). However, there was a significant treatment effect on leg muscle succinate (two-way ANOVA; P=0.012), where in *Vibrio*-injected crabs succinate was significantly higher immediately after walking than in saline-injected crabs (HS; P<0.05; Table 1).

Within leg muscles at rest, concentrations of arginine phosphate in both treatment groups were higher than those for free arginine. This corresponds to an arginine charge of 0.66 for saline-injected crabs and 0.57 for *Vibrio*-injected crabs (Table 1). During activity, arginine phosphate was readily dephosphorylated into arginine, causing arginine phosphate concentrations to drop significantly in saline- and *Vibrio*-injected crabs (one-way ANOVA with HS; P<0.01 for both treatments) with a corresponding significant increase in free arginine (HS; P<0.01; Table 1). After activity, arginine levels exceeded arginine phosphate levels, causing the arginine charge to significantly drop to approximately 0.4 in both treatments (Table 1; one-way ANOVA with HS; P<0.001 for both treatments). Both arginine and arginine phosphate levels returned to pre-activity levels within 1 h of recovery, reflected by an increase in arginine charge to approximately 0.7 for both treatments, and were indistinguishable from pre-activity levels (Table 1). There were no significant differences between saline-injected and *Vibrio*-injected crabs in phosphagen store measurements over time.

Although there is an apparent fluctuation of ATP and ADP levels in response to activity in both treatment groups, the

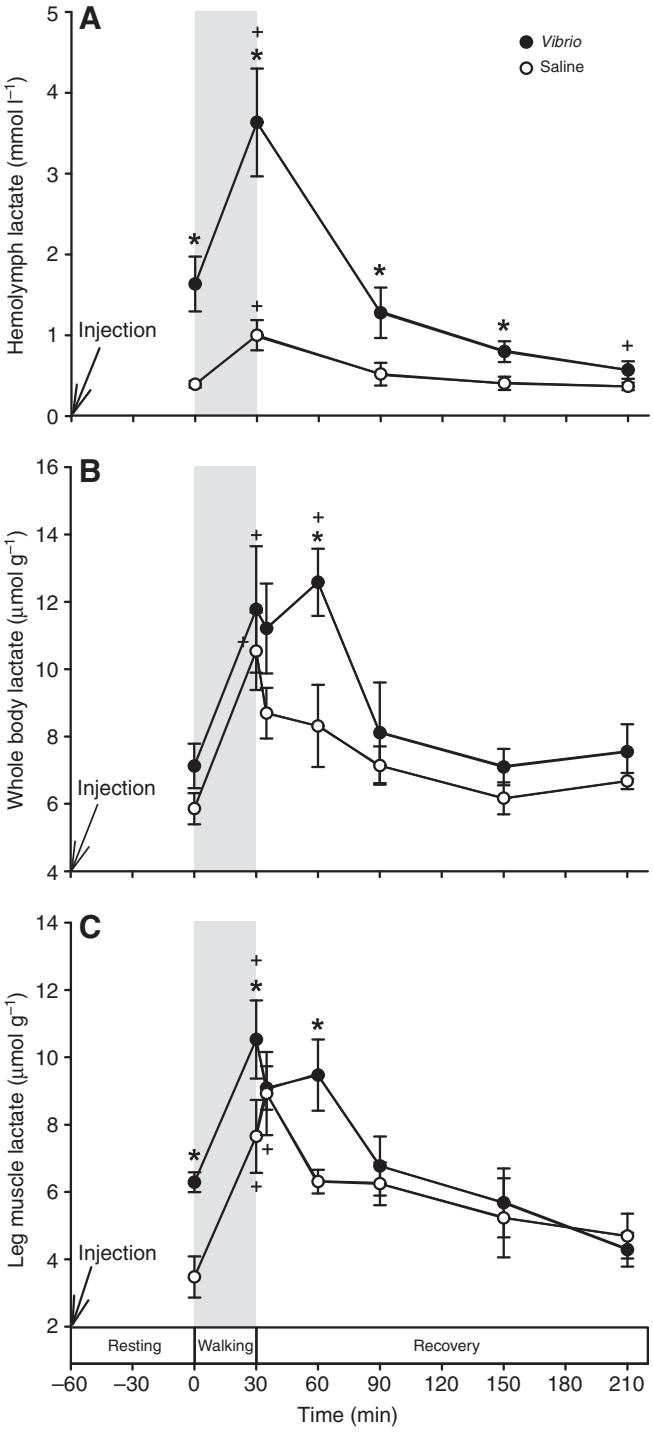

Fig. 5. Concentration of lactate in (A) hemolymph (mmol l^{-1}), (B) whole body (μmol g^{-1} tissue), and (C) leg muscle (μmol g^{-1} tissue) and in *C. sapidus* at rest 1 h following injection of *V. campbellii* (closed circles) or saline (control; open circles), immediately after 30 min of walking at 8 m min^{-1} and during a 3 h recovery from activity. Values are means ± s.e.m. *N*=5–9 for each treatment group. Whole body lactate, muscle lactate and hemolymph lactate all increased significantly over time in both saline-injected crabs and *Vibrio*-injected crabs (one-way ANOVA; $P<0.05$ for each measurement within each treatment). There was also a significant effect of injection treatment on whole body lactate, muscle lactate and hemolymph lactate during activity and recovery (two-way ANOVA; $P<0.05$ for each measurement). A plus sign indicates a significant increase in lactate compared with resting values within each treatment (HS; $P<0.05$). *A significant difference in lactate between treatments at individual timepoints (HS; $P<0.05$).

concentrations were not significantly different from resting values at any time during or after activity (Table 2). However, there was a significant change in AMP levels over time in saline-injected crabs (one-way ANOVA; $P=0.027$). AMP levels significantly increased with activity in saline-injected crabs and returned to pre-activity levels within 1 h of recovery (HS; $P<0.01$; Table 2). There was no difference in ATP or ADP concentrations between treatment groups at any timepoint. However, significantly higher AMP levels were found in *Vibrio*-injected crabs at rest (two-way ANOVA with HS; $P<0.02$). The adenylate energy charge, representing the overall energy status of the muscle, was high at rest (0.89±0.01) but significantly decreased with exercise to 0.77±0.03 (Table 2; two-way ANOVA with HS; $P<0.001$). Energy charge quickly returned to pre-activity levels within 1 h following exercise. There were no treatment differences in adenylate energy charge values before or after exercise.

DISCUSSION

Large numbers of *Vibrio* spp. are often detected in dead or moribund blue crabs (Krantz et al., 1969; Johnson, 1976; Welsh and Sizemore, 1985). However, *Vibrio* are often cultured from the hemolymph of apparently healthy blue crabs (e.g. Davis and Sizemore, 1982) and shrimp (e.g. Saulnier et al., 2000). These observations suggest that most *Vibrio* spp. act as opportunistic pathogens. The pathogenicity of *V. campbellii* is similar in *C. sapidus* (LD_{50}=6.2×10^5 CFU g^{-1} crab) and the Pacific whiteleg shrimp, *L. vannamei* (LD_{50}=3.06×10^5 CFU g^{-1} animal), but somewhat higher (LD_{50}=6.08×10^4 CFU g^{-1} animal) in the grass shrimp *Palaemonetes pugio*. This difference in virulence may be attributed to species-specific differences among hosts. Importantly, the LD_{50} data reported here for the blue crab document that the dose of 2.5×10^4 CFU g^{-1} animal used for immunological challenge experiments (Holman et al., 2004; Burgents et al., 2005b) (and the present study) is well below the LD_{50} value of *V. campbellii* in crabs and shrimp.

A sub-lethal dose of *V. campbellii* has a significant impact on resting aerobic metabolism, causing a 30% reduction in oxygen uptake 4 h after injection, and a more substantial 42% reduction in oxygen uptake at 24 h (Fig. 2). These results are similar to those of Scholnick et al. (Scholnick et al., 2006) who found that the shrimp *L. vannamei* experienced a 27% reduction in oxygen uptake 4 h after injection of *V. campbellii*, which declined 24 h later to 40%. Reduction of aerobic metabolism is accompanied by a small, but significant, increase in anaerobic metabolism as indicated by elevated hemolymph lactate levels (Fig. 3). The significant elevation of lactate in the hemolymph was not evident until 2 h following bacterial injection. It is possible that these small lactate changes could be indirect results of changes in hemolymph pH (not measured) which may alter the dynamic equilibrium between lactate and pyruvate. The result is an overall metabolic depression similar to the one observed in the Pacific white shrimp (Scholnick et al., 2006).

The metabolic depression appears to persist during walking exercise (Fig. 4) when oxygen demand rises. In these experiments, crabs walked 240 m at a speed of 8 m min^{-1} and in all cases did this without difficulty. This is not surprising since this species is capable of swimming at speeds of 7 m min^{-1} for at least 30 min (Houlihan et al., 1985) and in the wild displays rapid directional movements over long distances (Spirito, 1972; Wolcott and Hines, 1989; Hines et al., 1995).

The factorial metabolic scope of activity, defined as the ratio of active to resting oxygen uptake (Full and Herreid, 1983) is rather small, ranging from two to seven in most decapod crustaceans

Table 1. Anaerobic metabolite concentration in *C. sapidus* tissues (leg muscle and whole body) 1 h following injection of *V. campbellii* or saline (control) and after walking for 30 min at 8 m min^{-1}

Metabolic component	Treatment	Pre-exercise	Post-exercise 0 min	5 min	30 min	60 min	120 min	180 min
Arginine phosphate (leg muscle)	Control	29.6±4.1	20.4±2.4	12.8±1.7†	18.6±2.6†	24.3±1.3	24.3±2.4	32.6±2.6
	Vibrio	26.2±1.8	17.8±1.4	17.7±1.7†	22.5±2.2†	28.6±1.8	27.8±1.7	32.9±2.9
Arginine (leg muscle)	Control	15.0±1.7*	23.5±0.3†	23.3±1.1†	20.9±1.8	19.6±1.6	14.2±2.4	13.1±2.9
	Vibrio	20.0±1.4*	24.7±1.0†	22.6±1.2	22.4±1.9	15.5±1.1	16.5±2.4	16.4±1.7
Arginine charge (leg muscle)	Control	0.66±0.04	0.46±0.03†	0.35±0.03†	0.46±0.04†	0.56±0.02	0.64±0.05	0.72±0.06
	Vibrio	0.57±0.03	0.42±0.03†	0.44±0.02	0.50±0.04	0.65±0.07	0.63±0.04	0.66±0.04
Succinate (leg muscle)	Control	0.71±0.08	0.69±0.07*					
	Vibrio	0.86±0.07	0.96±0.07*					
Succinate (whole body)	Control	0.58±0.08	0.80±0.11					
	Vibrio	0.71±0.10	0.81±0.07					

Metabolite concentrations are in μmol g^{-1} tissue expressed as the mean±s.e.m.; *N*=6.

*Significant difference between treatment groups at each timepoint (two-way ANOVA with HS; $P<0.02$).

†Significant increase or decrease in post-exercise values compared to pre-exercise values within each treatment (one-way ANOVA with Dunnett's test; $P<0.001$).

(McMahon et al., 1979; Herreid, 1981; McMahon, 1981; Booth et al., 1982; Full and Herreid, 1984; Houlihan and Innes, 1984). During walking, O_2 uptake significantly increased ~2.5-fold and reached steady state within the first 10 min of activity (Fig. 4). This particular aerobic pattern is similar to that found in swimming blue crabs, indicating that blue crabs largely rely on aerobic ATP production during these activities (Booth et al., 1982). An aerobic scope of ~2.5 is surprisingly small and probably does not represent the maximal oxygen uptake for blue crabs (McMahon, 1981; Booth and McMahon, 1992). Indeed we chose a level of activity that blue crabs in both treatment groups could complete, but that clearly challenged the O_2 delivery system. Despite having efficient mechanisms to enhance oxygen uptake during increased activity, crabs infected with *Vibrio* did not increase oxygen uptake to levels measured in control animals (Fig. 4). These results are consistent with the idea that bacterial exposure impairs respiratory function in the blue crab because of the formation of hemocyte aggregates or nodules that become trapped in the gills (Johnson, 1976; Smith and Ratcliffe, 1980a; Martin et al., 2000; Burnett et al., 2006; Scholnick et al., 2006).

The excess post-exercise oxygen consumption (EPOC) during recovery in both treatments is partially due to energetic costs incurred during activity. EPOC is used to re-cycle or remove activity-induced buildup of lactate and replenish energy and oxygen stores used during sustained activity but could also be used for energetic demands of elevated ventilation and circulation that continues for some time after activity is completed (Herreid, 1981; Gaessar and Brooks, 1984; Gleeson and Hancock, 2001; Hancock and Gleeson, 2002). A relatively small O_2 deficit associated with a rapid recovery (<1 h) after walking in blue crabs is consistent with results found for swimming blue crabs and other running terrestrial crabs (Houlihan and Innes, 1984; Houlihan et al., 1984; Houlihan et al., 1985; Hamilton and Houlihan, 1992). The EPOC associated with walking was relatively small for all crabs but *Vibrio*-injected crabs (4.11 μmol O_2 g^{-1} crab) had a comparatively smaller EPOC than control crabs (6.51 μmol O_2 g^{-1} crab).

Although an aerobic steady state was reached in walking crabs, anaerobic metabolism was clearly used to generate some energy (Fig. 5A–C). This could be a consequence of a limitation of oxygen supply to the gills or of mild muscle hypoxia induced by a limitation of oxygen delivery at the tissues (McMahon, 1981; Burnett et al., 2006). Resting whole body lactate was high (~6 μmol g^{-1}) compared with resting levels of 1.2 μequiv g^{-1} previously found in blue crabs (Booth and McMahon, 1985). A twofold increase in whole body lactate during 30 min of walking (Fig. 5B) is modest compared with a 6.1-fold increase in blue crabs that swam for 30 min (Booth and McMahon, 1985). Blue crabs were able to eliminate excess whole body lactate in less than 2 h after walking (Fig. 5B), which is much

Table 2. Adenylate concentration and associated adenylate energy charge in the leg muscle of *C. sapidus* at rest 1 h following injection of *V. campbellii* or saline (control) and after walking for 30 min at 8 m min^{-1}

Adenylate measure	Treatment	Pre-exercise	Post-exercise 0 min	5 min	30 min	60 min	120 min	180 min
ATP	Control	5.70±0.49	4.36±0.53	4.72±0.41	4.86±0.69	5.46±0.52	5.36±0.68	5.58±0.92
	Vibrio	6.00±0.59	4.76±0.50	3.92±0.75	5.00±0.52	5.93±0.54	6.10±0.80	6.03±0.52
ADP	Control	1.15±0.28	1.71±0.29	1.49±0.38	1.45±0.19	1.40±0.22	0.99±0.24	1.23±0.16
	Vibrio	1.31±0.14	1.62±0.14	1.37±0.17	1.30±0.25	1.47±0.21	1.30±0.29	1.33±0.10
AMP	Control	0.20±0.04*	0.66±0.15†	0.62±0.11†	0.56±0.13†	0.38±0.09	0.35±0.06	0.41±0.06
	Vibrio	0.47±0.09*	0.58±0.16	0.56±0.14	0.47±0.08	0.27±0.05	0.33±0.09	0.28±0.05
Energy charge	Control	0.89±0.01	0.77±0.03†	0.80±0.02†	0.81±0.03	0.85±0.01†	0.87±0.01	0.86±0.01
	Vibrio	0.85±0.02	0.80±0.02	0.76±0.05	0.83±0.02	0.86±0.02	0.86±0.03	0.87±0.01

Adenylate concentrations are in μmol g^{-1} tissue expressed as the mean±s.e.m.; *N*=6.

*Significant difference between treatment groups at each timepoint (two-way ANOVA with HS; $P<0.05$).

†Significant increase or decrease in post-exercise values compared to pre-exercise values within each treatment (one-way ANOVA with Dunnett's test; $P<0.03$).

shorter than the recovery period (~8 h) for swimming blue crabs (Booth and McMahon, 1985). Levels of lactate in leg muscles were similar in value and pattern to whole body lactate during and after walking, suggesting that whole body lactate measurements are representative of lactate levels present in muscle tissue (Fig. 5B,C).

At least some of the lactate that builds up in active muscle during activity is released into the hemolymph (Fig. 5A). Resting hemolymph lactate (<2 mmol l^{-1}) was similar to levels measured previously in the blue crab (Booth et al., 1982; Booth and McMahon, 1985; Gannon and Wheatly, 1995; Johnson et al., 2004). A modest 2.2- to 2.5-fold increase in hemolymph lactate in crabs that had walked for 30 min was comparable to two- to fourfold increases reported for blue crabs after swimming bouts of 15 min (Gannon and Wheatly, 1995) but less than the 14- to 15-fold increase reported after swimming for 25 min (Booth et al., 1982) and 30 min (Booth and McMahon, 1985; Milligan et al., 1989) and 1 h (7-fold increase) (Booth et al., 1984).

Differences in O_2 uptake during walking between control and *Vibrio*-challenged crabs were not offset by the differences in lactate production. However, blue crabs could also be excreting lactate into the environment (de Zwaan and Skjoldal, 1979; Head and Baldwin, 1986) or producing other anaerobic end products, such as succinate. Generation of succinate is a high efficiency–low energy output pathway that is most often used by invertebrates during periods of environmental anaerobiosis (Livingstone, 1991). However, succinate levels in whole body and muscle were considerably lower than those of lactate in both tissue types and changed very little with injection treatment and with strenuous activity, suggesting that succinate production and, probably, anaerobic ATP-generating pathways overall, do not play an important role in *Vibrio*-associated changes in metabolism (Table 1).

Only a small number of studies have documented adenylate and phosphagen usage within crustacean muscles. In the walking leg muscle, arginine phosphate was found in high concentrations (25–30 μmol g^{-1}) along with comparatively lower concentrations of arginine (15–20 μmol g^{-1}; Table 1). This corresponds to a resting arginine charge of 0.66, which is comparable to 0.69 found in blue crab levator muscle (Hardy et al., 2006) and 0.59 in crayfish leg muscle (Speed et al., 2001) but lower than 0.8 in red crab leg muscle (Morris and Adamczewska, 2002). These slight differences are mostly due to varied concentrations of free arginine present in the muscle. Arginine phosphate in blue crab leg muscle (29.6 μmol g^{-1}; Table 1) was similar to that found in blue crab levator muscle (29.9 μmol g^{-1}) (Hardy et al., 2006) and in spiny lobster leg muscle (31.35 μmol g^{-1}) (Speed et al., 2001), but higher than concentrations found in highly aerobic ghost crabs (21.5 μmol g^{-1}) (Full and Weinstein, 1992). ATP content (5.7 μmol g^{-1}; Table 2), indicating muscle energetic condition, was comparable to concentrations in red crab leg muscle (4.76 μmol g^{-1}) (Morris and Adamczewska, 2002) and in spiny lobster leg muscle (5.14 μmol g^{-1}) (Speed et al., 2001) but lower than ATP content measured in crayfish tail muscle (7.43 μmol g^{-1}) (Head and Baldwin, 1986) and in blue crab levator muscle (7.3 μmol g^{-1}) (Hardy et al., 2006). The resting adenylate energy charge in blue crab leg muscle (0.89) was high (Table 2) but very comparable to the energy charge found in other crustacean muscles (England and Baldwin, 1983; Speed et al., 2001; Morris and Adamczewska, 2002).

High concentrations of arginine phosphate and ATP are generally associated with muscle fibers that predominantly use anaerobic energy production (England and Baldwin, 1983; Head and Baldwin, 1986; Speed et al., 2001). Although the muscle tissue analyzed here most likely contained a combination of aerobic and anaerobic muscle fibers and no attempt was made to distinguish between the two fiber types, phosphagen energy stores are clearly important during walking as shown by a pronounced 57% depletion in arginine phosphate associated with a 55% increase in free arginine, causing a dramatic drop in arginine charge from 0.66 to 0.35 (Table 1). This large drop in the phosphagen energy stores appears to protect ATP content, which did not significantly change with increased activity (Table 2). This response seems like a general strategy used during muscle oxygen deficiency in crustaceans (Hill et al., 1991). Although only a small fluctuation in ATP levels occurred in response to 30 min walking, the adenylate energy charge (AEC) decreased by 13% during activity mostly due to an increase in AMP levels (Table 2). This indicated that the energetic demands of walking exceeded the buffering capacity of arginine phosphate stores. However, the energetic demands on blue crab walking leg muscle do not seem to be as extreme as the requirements for more intense muscular activity, such as those for crayfish tail muscle after simulated escape behavior, which caused a 28% drop in AEC after exhaustive tail flipping (England and Baldwin, 1983).

The steady state O_2 uptake at a particular work load, converted into energy ATP equivalents, is usually taken as the total energetic cost of that particular activity in highly aerobic vertebrates (reviewed by Herreid, 1981), but a mixture of both aerobic and anaerobic responses are generally reported for crustaceans. We estimated the energetic cost of 30 min of walking by converting the measurement of each metabolic component to ATP energy equivalents (Table 3). Energy production during activity is associated with oxygen consumption (1 mol O_2=6.33 mol ATP), lactate accumulation (1 mol lactate=1.5 mol ATP) and net arginine phosphate and ATP use (1 mol arginine phosphate=1 mol ATP); these are assumptions based on glycogen as the main fuel source (Hohnke and Scheer, 1970; England and Baldwin, 1983). Although aerobic ATP production accounted for the majority (74–80%) of the calculated energetic cost during 30 min of walking, only 15–20% was supported by anaerobic metabolism (Table 3). Although we conservatively assumed that a steady accumulation of lactate was occurring over the 30 min activity period, this contribution is probably most important during the first few minutes of activity. Less than 5% of the total cost was attributed to net arginine phosphate and net ATP use (Table 3). The total energetic cost of exercise in *Vibrio*-injected crabs (68.0 μmol ATP g^{-1} h^{-1}) was 74% of the total cost estimated for saline-injected crabs (91.6 μmol ATP g^{-1} h^{-1}). This difference in energetic cost is almost entirely due to a reduced contribution of aerobic ATP production to the total energy cost.

The general lack of a large recruitment of anaerobic metabolism and stability of AEC during stress are defining characteristics of metabolic depression (Guppy et al., 1994; Reipschlager and Pörtner,

Table 3. The relative contribution to the total energetic cost during 30 min walking of oxygen uptake, lactate production, net arginine phosphate and ATP use

Metabolic component	Control	*Vibrio*
Oxygen uptake	73.4	50.3
Lactate production	14	13.9
Arginine phosphate use	4.6	4.2
ATP use	0.7	0.6
TOTAL	**92.7**	**69.0**

Values were calculated as μmol ATP equivalents g^{-1} h^{-1}.
Net use or accumulation was assumed to occur over the entire 30 min walking period. Arginine phosphate and ATP depletion were calculated on a per gram total body mass basis with the assumption that a typical crab contains 25% locomotory muscle mass (Herreid, 1981).

1996). We found only a 5% increase in anaerobic metabolism in response to *Vibrio* injection to compensate for the large reduction in aerobic metabolism (Table 3). Additionally, there were no differences in arginine and adenylate energy charge (Tables 1 and 2). These results together support the hypothesis of a metabolic depression induced by injection of bacteria that persists during increased activity.

The phenomenon of metabolic depression has been documented in the majority of major animal phyla (reviewed by Guppy and Withers, 1999; Storey and Storey, 2004). Many invertebrates are capable of decreasing overall metabolism in response to environmental stressors such as hypoxia, desiccation and extreme cold (Guppy et al., 1994; Burnett, 1997; Guppy, 2004). Metabolic depression may also be a stress response in crustaceans exposed to bacteria. Although the actual systemic bacterial infection itself may cause a metabolic depression to occur, this possibility is unlikely since culturable bacteria are rapidly cleared from the hemolymph and other major tissues in blue crabs and penaeid shrimp (Holman et al., 2004; Burgents et al., 2005b). Also, we cannot rule out the possibility that there may be direct or indirect effects of the bacteria itself, its associated endotoxins and exotoxins or hormones that are released in response to bacterial stress. For example, bacterial lipopolysaccharide not only induces the normal hemocytopenic response but also the release of crustacean hyperglycemic hormone, a hormone that is considered the primary regulator of metabolic fuel availability in crustaceans and can cause multiple physiological effects in moderately stressed organisms (Lorenzon et al., 1997; Lorenzon et al., 1999; Morris, 1999; Fanjul-Moles, 2006). Therefore, it is possible that reduction of oxygen uptake in response to bacterial exposure may be hormonally controlled, as has been found in respiration and ionic regulation in the hololimnetic shrimp *Macrobrachium potiuna* in response to salinity stress (de Souza and Moreira, 1994).

Animals experiencing a metabolic depression generally show decreased overall activity (Storey and Storey, 1990). However, *Vibrio*-injected crabs along with their controls were clearly able to accomplish walking exercise. The difference in metabolic rate between the control and *Vibrio*-treated crabs during exercise is nearly identical to the metabolic depression observed in resting crabs. This result was unexpected. *Vibrio*-injected crabs were able to walk for 30 min and they were clearly able to do this metabolically in a manner similar to control crabs (Table 3). We had expected that *Vibrio*-injected crabs would find it more difficult to deliver the increased oxygen required during exercise if their oxygen transport system were truly impaired (Burnett et al., 2006); however, these experiments were conducted in well-aerated water and we suspect that the results would be quite different in hypoxic conditions.

Although metabolic depression could indicate a reduction in all cellular processes, we hypothesize that a reduction in metabolism is probably due to decreases in protein synthesis rate and the associated ATP costs of protein turnover (Storey and Storey, 1990; Storey and Storey, 2004). Protein synthesis is an energy-expensive process and can account for as much as 37% of aerobic metabolism in crustaceans (Houlihan et al., 1990). Indeed, a significant reduction in protein synthesis during metabolic depression has been reported in numerous organisms and is frequently associated with the liver and hepatopancreas (Hochachka et al., 1996; Fuery et al., 1998; Pakay et al., 2002).

The mechanisms by which priorities of energy expenditure are re-arranged to sustain necessary functions while under stress are unclear. Interestingly, activation of AMP-activated protein kinase (AMPK), a 'metabolic master switch' in mammals that acts to preserve ATP during stress by increasing ATP generating pathways (glycolysis, fatty acid oxidation, glucose uptake) and decreasing ATP consuming pathways (synthesis of fatty acids, cholesterol and protein), occurs in response to depleted cellular energy pools that occur during prolonged exercise and hypoxic stress (Winder and Hardie, 1999; Kemp et al., 2002). AMPK has recently been identified as a central mediator of the translational control of protein synthesis during hypoxic stress in rat hepatocytes (Horman et al., 2002; Kemp et al., 2002). It has now been confirmed that AMPK is present in crustaceans and is activated in response to both temperature and hypoxic stress (Pinz et al., 2005; Frederich et al., 2009). Therefore, AMPK, if present in the blue crab, may be playing a role in regulating energy metabolism during bacterial stress.

Despite the fact that the total energy expenditure during walking in *Vibrio*-exposed crabs accounted for only 74% of the energy expenditure in saline-injected blue crabs, the decrease in the amount of ATP produced by aerobic means does not appear to affect the overall performance of crabs during moderate walking activity. A crab's ability to walk continuously for 30 min at a moderate pace is not greatly affected by *Vibrio* injection. However, crabs infected with bacteria are less aerobic while doing so, as are resting crabs. While movement in optimal environmental conditions may not pose much of a problem, should that crab encounter hypoxic waters or some other stressful environmental condition, reduced metabolism associated with bacterial infection may render the animal less fit to meet the demands of normal activity necessary for survival in the wild.

Regardless of the mechanisms by which aerobic metabolism is reduced, significant metabolic adjustments that occur after bacterial stress would surely be intensified by the effects of environmental hypoxia or high levels of animal activity. Hypoxic conditions occur in coastal and estuarine environments and are increasing on a global level (Diaz and Rosenberg, 2008). Blue crabs are able to tolerate external hypoxic conditions by compensating with behavioral, metabolic and circulatory adjustments (Batterton and Cameron, 1978; deFur et al., 1990; Burnett, 1997; Mangum, 1997; Burnett and Stickle, 2001) but environmental hypoxia itself negatively impacts immune function (Holman et al., 2004; Burgents et al., 2005a; Macey et al., 2008). Blue crabs are also able to perform high levels of activity necessary for survival in the field (Spirito, 1972; Booth et al., 1982). In cases of both hypoxia and increased activity, the oxygen delivery system fails to completely supply the necessary energy needed, and is supplemented by anaerobic pathways producing lactic acid (Booth et al., 1982; Booth and McMahon, 1985; Burnett and Stickle, 2001), in a manner similar to that when a blue crab is experimentally infected with bacteria. Dealing with either of these additional stressful situations while simultaneously fighting a bacterial infection would only further affect the already-compromised metabolic status of an animal and possibly its survival in the field.

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REFERENCES

Adams, A. (1991). Response of penaeid shrimp to exposure to *Vibrio* species. *Fish Shellfish Immunol.* **1**, 59-70.

Atkinson, D. E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **7**, 4030-4034.

Bartholomew, G. A., Vleck, D. and Vleck, C. M. (1981). Instantaneous measurements of oxygen consumption during pre-flight warm-up and post-flight cooling in sphingid and saturniid moths. *J. Exp. Biol.* **90**, 17-32.

Batterton, C. V. and Cameron, J. N. (1978). Characteristics of resting ventilation and response to hypoxia, hypercapnia, and emersion in the blue crab *Callinectes sapidus* (Rathbun). *J. Exp. Zool.* **203**, 403-418.
Bergmeyer, H. U. (1985a). Metabolites 3: lipids, amino acids and related compounds. In *Methods of Enzymatic Analysis*, vol. 8 (ed. H. U. Bergmeyer, J. Bergmeyer and M. Grassl). Weinheim: VCH.
Bergmeyer, H. U. (1985b). Metabolites 2: tri and dicarboxylic acids, purines, pyrimidines and derivatives, coenzymes, inorganic compounds. In *Methods of Enzymatic Analysis*, vol. 7 (ed. H. U. Bergmeyer, J. Bergmeyer and M. Grassl). Weinheim: VCH.
Booth, C. E. and McMahon, B. R. (1985). Lactate dynamics during locomotor activity in the blue crab *Callinectes sapidus*. *J. Exp. Biol.* **118**, 461-465.
Booth, C. E. and McMahon, B. R. (1992). Aerobic capacity of the blue crab, *Callinectes sapidus*. *Physiol. Zool.* **65**, 1074-1091.
Booth, C. E., McMahon, B. R. and Pinder, A. W. (1982). Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab *Callinectes sapidus*. *J. Comp. Physiol.* **148**, 111-121.
Booth, C. E., McMahon, B. R., deFur, P. L. and Wilkes, P. R. H. (1984). Acid–base regulation during exercise and recovery in the blue crab, *Callinectes sapidus*. *Respir. Physiol.* **58**, 359-376.
Burgents, J. E., Burnett, K. G. and Burnett, L. E. (2005a). Effects of hypoxia and hypercapnic hypoxia on the localization and the elimination of *Vibrio campbellii* in *Litopenaeus vannamei*, the Pacific white shrimp. *Biol. Bull.* **208**, 159-168.
Burgents, J. E., Burnett, L. E., Stabb, E. V. and Burnett, K. G. (2005b). Localization and bacteriostasis of *Vibrio* introduced into the Pacific white shrimp, *Litopenaeus vannamei*. *Dev. Comp. Immunol.* **29**, 681-691.
Burnett, L. E. (1997). The challenges of living in hypoxic and hypercapnic aquatic environments. *Am. Zool.* **37**, 633-640.
Burnett, L. E. and Stickle, W. B. (2001). Physiological responses to hypoxia. In *Coastal Hypoxia: Consequences for Living Resources and Ecosystems, Coastal and Estuarine Studies*, vol. 57 (ed. N. N. Rabalais and R. E. Turner), pp. 101-114. Washington DC: American Geophysical Union.
Burnett, L. E., Holman, J. D., Jorgensen, D. D., Ikerd, J. L. and Burnett, K. G. (2006). Immune defense reduces respiratory fitness in *Callinectes sapidus*, the Atlantic blue crab. *Biol. Bull.* **211**, 50-57.
Cochran, D. M. (1935). The skeletal musculature of the blue crab, *Callinectes sapidus* Rathbun. *Smithson. Misc. Collect.* **92**, 1-76.
Colwell, R. R., Wicks, T. C. and Tubiash, H. S. (1975). A comparative study of the bacterial flora of the hemolymph of *Callinectes sapidus*. *Mar. Fish. Rev.* **37**, 29-33.
Davis, J. W. and Sizemore, R. K. (1982). Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas. *Appl. Environ. Microbiol.* **43**, 1092-1097.
deFur, P. L., Mangum, C. P. and Reese, J. E. (1990). Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia. *Biol. Bull.* **178**, 46-54.
de Souza, S. C. R. and Moreira, G. S. (1994). Neuroendocrine influence on whole animal and tissue respiration and on osmoionic regulation in the hololimnetic shrimp *Macrobrachium potiuna* (Müller, 1880). *J. Crust. Biol.* **14**, 36-46.
de Zwaan, A. and Skjoldal, H. R. (1979). Anaerobic energy metabolism of the scavenging isopod *Cirolana borealis* (Lilljeborg). *Comp. Biochem. Physiol. B* **129**, 327-331.
Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-929.
England, W. R. and Baldwin, J. (1983). Anaerobic energy metabolism in the tail musculature of the Australian yabby *Cherax destructor* (Crustacea, Decapoda, Parastacidae): role of phosphagens and anaerobic glycolysis during escape behavior. *Physiol. Zool.* **56**, 614-622.
Fanjul-Moles, M. L. (2006). Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: review and update. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **142**, 390-400.
Frappell, P. B., Blevin, H. A. and Baudinette, R. V. (1989). Understanding respirometry chambers: what goes in must come out. *J. Theor. Biol.* **138**, 479-485.
Frederich, M., O'Rourke, M. R., Furey, N. B. and Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *J. Exp. Biol.* **212**, 722-730.
Fuery, C. J., Withers, P. C., Hobbs, A. A. and Guppy, M. (1998). The role of protein synthesis during metabolic depression in the Australian desert frog *Neobatrachus centralis*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **119**, 469-476.
Full, R. J. and Herreid, C. F. (1983). Aerobic response to exercise of the fastest land crab. *Am. J. Physiol.* **244**, R530-R536.
Full, R. J. and Herreid, C. F. (1984). Fiddler crab exercise: the energetic cost of running sideways. *J. Exp. Biol.* **109**, 141-161.
Full, R. J. and Weinstein, R. B. (1992). Integrating the physiology, mechanics and behavior of rapid running ghost crabs: slow and steady doesn't always win the race. *Am. Zool.* **32**, 382-395.
Gaessar, G. A. and Brooks, G. A. (1984). Metabolic bases of excess post-exercise oxygen consumption: a review. *Med. Sci. Sports Exerc.* **15**, 29-43.
Gannon, A. T. and Wheatly, M. G. (1995). Physiological effects of a gill barnacle on host blue crabs during short-term exercise and recovery. *Mar. Behav. Physiol.* **24**, 215-222.
Gleeson, R. A. and Zubkoff, P. L. (1977). The determination of hemolymph volume in the blue crab, *Callinectes sapidus*, utilizing ^{14}C-thiocyanate. *Comp. Biochem. Physiol.* **56A**, 411-413.
Gleeson, T. T. and Hancock, T. V. (2001). Modeling the metabolic energetics of brief and intermittent locomotion in lizards and rodents. *Am. Zool.* **41**, 211-218.
Graham, R. A., Mangum, C. P., Terwilliger, R. C. and Terwilliger, N. B. (1983). The effect of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister*. *Comp. Biochem. Physiol. A Physiol.* **74**, 45-50.
Guppy, M. (2004). The biochemistry of metabolic depression: a history of perceptions. *Comp. Biochem. Physiol.* **139B**, 435-442.
Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev.* **74**, 1-40.
Guppy, M., Fuery, C. J. and Flanigan, J. E. (1994). Biochemical principles of metabolic depression. *Comp. Biochem. Physiol B Biochem. Mol. Biol.* **109**, 175-189.
Hamilton, M. A., Russo, R. C. and Thurston, R. V. (1977). Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.* **11**, 714-719.
Hamilton, N. M. and Houlihan, D. F. (1992). Respiratory and circulatory adjustments during aquatic treadmill exercise in the European shore crab *Carcinus maenas*. *J. Exp. Biol.* **162**, 37-54.
Hancock, T. V. and Gleeson, T. T. (2002). Metabolic recovery in the Desert Iguana (*Dipsosaurus dorsalis*) following activities of varied intensity and duration. *Funct. Ecol.* **16**, 40-48.
Hardy, K. M., Locke, B. R., Da Silva, M. and Kinsey, S. T. (2006). A reaction-diffusion analysis of energetics in large muscle fibers secondarily evolved for aerobic locomotor function. *J. Exp. Biol.* **209**, 3610-3620.
Head, C. and Baldwin, J. (1986). Energy metabolism and the fate of lactate during recovery from exercise in the Australian freshwater crayfish *Cherax destructor*. *Aust. J. Mar. Freshw. Res.* **37**, 641-646.
Herreid, C. F. (1981). Energetics of pedestrian arthropods. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid and C. R. Fourtner). New York: Plenum Press.
Herreid, C. F., Lee, L. W. and Shah, G. M. (1979). Respiration and heart rate in exercising land crabs. *Respir. Physiol.* **36**, 109-120.
Hill, A. D., Taylor, A. C. and Strang, R. H. C. (1991). Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery. *J. Exp. Mar. Biol. Ecol.* **150**, 31.
Hines, A. H. (2007). Ecology of juvenile and adult blue crabs. In *The Blue Crab Callinectes sapidus* (ed. V. S. Kennedy and E. Cronin), pp. 565-654. College Park, MD: Maryland Sea Grant College.
Hines, A. H., Wolcott, T. G., Gonzalez-Gurriaran, E., Gonzalez-Escalante, J. L. and Friere, J. (1995). Movement patterns and migrations in crabs: Telemetry of juvenile and adult behaviour in *Callinectes sapidus* and *Maja squinado*. *J. Mar. Biol. Assoc. UK* **75**, 27-42.
Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.
Hohnke, L. and Scheer, B. T. (1970). Carbohydrate metabolism in crustaceans. In *Chemical Zoology*, vol. 5 (ed. M. Florkin and B. T. Scheer), 460pp. New York: Academic Press.
Holman, J. D., Burnett, K. G. and Burnett, L. E. (2004). Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic blue crab, *Callinectes sapidus* Rathbun. *Biol. Bull.* **206**, 188-196.
Horman, S., Browne, G. J., Krause, U., Patel, J. V., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. G. and Rider, M. H. (2002). Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr. Biol.* **12**, 1419-1423.
Houlihan, D. F. and Innes, A. J. (1984). The cost of walking in crabs: aerial and aquatic oxygen consumption during activity of two species of intertidal crab. *Comp. Biochem. Physiol. A Physiol.* **77**, 325-334.
Houlihan, D. F., Mathers, E. and El Haj, A. J. (1984). Walking performance and aerobic and anaerobic metabolism of *Carcinus maenas* (L.) in sea water at 15 degrees C. *J. Exp. Mar. Biol. Ecol.* **74**, 211-230.
Houlihan, D. F., Govind, C. K. and El Haj, A. (1985). Energetics of swimming in *Callinectes sapidus* and walking in *Homarus americanus*. *Comp. Biochem. Physiol. A Physiol.* **82**, 267-279.
Houlihan, D. F., Waring, C. P., Mathers, E. and Gray, C. (1990). Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. *Physiol. Zool.* **634**, 735-756.
Johnson, L. K., Dillaman, R. M., Gay, D. M., Blum, J. E. and Kinsey, S. T. (2004). Metabolic influences of fiber size in aerobic and anaerobic locomotor muscles of the blue crab, *Callinectes sapidus*. *J. Exp. Biol.* **207**, 4045-4056.
Johnson, P. T. (1976). Bacterial infection in the blue crab, *Callinectes sapidus*: course of infection and histopathology. *J. Invertebr. Pathol.* **28**, 25-36.
Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murthy, S., Walter, M., Gupta, A., Adams, J. J., Katsis, F., van Denderen, B. et al. (2002). AMP-activated protein kinase, super metabolic regulator. *Biochem. Soc. Trans.* **31**, 162-168.
Krantz, G. F., Colwell, R. R. and Lovelace, E. (1969). *Vibrio parahaemolyticus* from the blue crab *Callinectes sapidus* in Chesapeake Bay. *Science* **164**, 1286-1287.
Le Moullac, G. and Haffner, P. (2000). Environmental factors affecting immune responses in Crustacea. *Aquaculture* **191**, 121-131.
Livingstone, D. R. (1991). Origins and evolution of pathways of anaerobic metabolism in the Animal Kingdom. *Am. Zool.* **31**, 522-534.
Lorenzon, S., Giulianini, P. G. and Ferrero, E. A. (1997). Lipopolysaccharide-induced hyperglycemia is mediated by CHH release in crustaceans. *Gen. Comp. Endocrinol.* **108**, 395-405.
Lorenzon, S., de Guarrini, S., Smith, V. J. and Ferrero, E. A. (1999). Effects of LPS injection on circulating haemocytes in crustaceans in vivo. *Fish Shellfish Immunol.* **9**, 31-50.
Macey, B. M., Rathburn, C. K., Thibodeaux, L. K., Burnett, L. E. and Burnett, K. G. (2008). Clearance of *Vibrio campbellii* injected into the hemolymph of *Callinectes sapidus*, the Atlantic blue crab: the effects of prior exposure to bacteria and environmental hypoxia. *Fish Shellfish Immunol.* **25**, 165-171.
Mangum, C. P. (1997). Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*. *Am. Zool.* **37**, 604-611.

Martin, G. G., Poole, D., Poole, C., Hose, J. E., Arias, M., Reynolds, L., McKrell, N. and Whang, A. (1993). Clearance of bacteria injected into the hemolymph of the penaeid shrimp, *Sicyonia ingentis*. *J. Invertebr. Pathol.* **62**, 308-315.
Martin, G. G., Quintero, M., Quigley, M. and Khosrovian, H. (2000). Elimination of sequestered material from the gills of decapod crustaceans. *J. Crustacean Biol.* **20**, 209-217.
McCumber, L. and Clem, W. L. (1977). Recognition of viruses and xenogeneic proteins by the blue crab, *Callinectes sapidus*. I. Clearance and organ concentration. *Dev. Comp. Immunol.* **1**, 5-14.
McDonald, D. G., McMahon, B. R. and Wood, C. M. (1979). An analysis of acid–base disturbances in the haemolymph following strenuous activity in the Dungeness crab, *Cancer magister*. *J. Exp. Biol.* **79**, 47-58.
McMahon, B. R. (1981). Oxygen uptake and acid-base balance during activity in decapod crustaceans. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid and C. R. Fourtner), pp. 299-335. New York: Plenum Press.
McMahon, B. R., McDonald, D. G. and Wood, C. M. (1979). Ventilation, oxygen uptake and haemolymph oxygen transport, following enforced exhausting activity in the Dungeness crab, *Cancer magister*. *J. Exp. Biol.* **80**, 271-285.
Merrill, D. P., Mongeon, S. A. and Fisher, S. (1979). Distribution of fluorescent latex particles following clearance from the hemolymph of the freshwater crayfish *Orconectes virilis* (Hagen). *J. Comp. Physiol.* **132**, 363-368.
Mikulski, C. M., Burnett, L. E. and Burnett, K. G. (2000). The effects of hypercapnic hypoxia on the survival of shrimp challenged with *Vibrio parahaemolyticus*. *J. Shellfish Res.* **19**, 301-311.
Milligan, C. L., Walsh, P. J., Booth, C. E. and McDonald, G. D. (1989). Intracellular acid–base regulation during recovery from locomotor activity in the blue crab (*Callinectes sapidus*). *Physiol. Zool.* **62**, 621-638.
Morris, S. (1999). Integration of physiological responses of crustaceans to environmental challenge. *South Afr. J. Zool.* **33**, 87.
Morris, S. and Adamczewska, A. M. (2002). Utilisation of glycogen, ATP and arginine phosphate in exercise and recovery in terrestrial red crabs, *Gecarcoidea natalis*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **133**, 813-825.
Morris, S., van Aardt, W. J. and Ahern, M. D. (2005). The effect of lead on the metabolic and energetic status of the Yabby, *Cherax destructor*, during environmental hypoxia. *Aquat. Toxicol.* **75**, 16-31.
Newman, M. C. and Feng, F. Y. (1982). Susceptibility and resistance of the rock crab, *Cancer irroratus*, to natural and experimental bacterial infection. *J. Invertebr. Pathol.* **40**, 75-88.
Pakay, J. L., Withers, P. C., Hobbs, A. A. and Guppy, M. (2002). In vivo downregulation of protein synthesis in the snail *Helix apersa* during estivation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R197-R204.
Pinz, I., Perry, D. J. and Fredrich, M. (2005). Activation of 5′-AMP activated protein kinase during anaerobiosis in the rock crab, *Cancer irroratus*. *MDIBL Bull.* **44**, 31-32.
Pugh, R. S., Ellisor, M. B., Moors, A. J., Porter, B. J. and Becker, P. R. (2007). Marine Environmental Specimen Bank: Clean Room and Specimen Bank Protocols.
Reipschlager, A. and Pörtner, H. O. (1996). Metabolic depression during environmental stress: the role of extracellular *versus* intracellular pH in *Sipunculus nudus*. *J. Exp. Biol.* **199**, 1801-1807.
Saulnier, D., Haffner, P., Goarant, C., Levy, P. and Ansquer, D. (2000). Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture* **191**, 133-144.
Scholnick, D. A., Burnett, K. G. and Burnett, L. E. (2006). Impact of exposure to bacteria on metabolism in the penaeid shrimp *Litopenaeus vannamei*. *Biol. Bull.* **211**, 44-49.
Schulte, P. M., Moyes, C. D. and Hochachka, P. W. (1992). Integrating metabolic pathways in post-exercise recovery of white muscle. *J. Exp. Biol.* **166**, 181-195.
Sizemore, R. K. and Davis, J. W. (1985). Source of *Vibrio* spp. found in the hemolymph of the blue crab, *Callinectes sapidus*. *J. Invertebr. Pathol.* **46**, 109-110.
Smith, V. J. and Ratcliffe, N. A. (1980a). Cellular defense reactions of the shore crab, *Carcinus maenas*: in vivo hemocytic and histopathological responses to injected bacteria. *J. Invertebr. Pathol.* **35**, 65-74.
Smith, V. J. and Ratcliffe, N. A. (1980b). Host defense reactions of the shore crab, *Carcinus maenas* (L.): clearance and distribution of injected test particles. *J. Mar. Biol. Assoc. UK* **60**, 89-102.
Speed, S. R., Baldwin, J., Wong, R. J. and Wells, R. M. G. (2001). Metabolic characteristics of muscles in the spiny lobster, *Jasus edwardsii*, and responses to emersion during simulated live transport. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **128**, 435-444.
Spirito, C. P. (1972). An analysis of swimming behavior in the portunid crab, *Callinectes sapidus*. *Mar. Behav. Physiol.* **1**, 261-276.
Storey, K. B. and Storey, J. M. (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145-174.
Storey, K. B. and Storey, J. M. (2004). Metabolic rate depression in animals: transcriptional and translational controls. *Biol. Rev.* **79**, 207-233.
Taylor, E. W. (1982). Control and co-ordination of ventilation and circulation in crustaceans responses to hypoxia and exercise. *J. Exp. Biol.* **100**, 289-319.
Towle, D. W. and Burnett, L. E. (2006). Osmoregulatory, digestive, and respiratory physiology. In *The Biology of the Blue Crab* (ed. V. Kennedy), pp. 1-31. Baltimore, MD: Maryland Sea Grant.
Tubiash, H. S., Sizemore, R. K. and Colwell, R. R. (1975). Bacterial flora of the hemolymph of the blue crab, *Callinectes sapidus*: most probably numbers. *Appl. Microbiol.* **29**, 388-392.
van de Braak, C. B., Botterblom, M. H., Taverne, N., van Muiswinkel, W. B., Rombout, J. H. and van der Knaap, W. P. (2002). The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. *Fish Shellfish Immunol.* **13**, 293-309.
Welsh, P. C. and Sizemore, R. K. (1985). Incidence of bacteremia in stressed and unstressed populations of the blue crab, *Callinectes sapidus*. *Appl. Environ. Microbiol.* **50**, 420-425.
Whitaker, J. D., Delancey, L. B., Jenkins, J. E. and Maddox, M. B. (1998). A review of the fishery and biology of the blue crab, *Callinectes sapidus*, in South Carolina. *J. Shellfish Res.* **17**, 459-463.
White, K. N. and Ratcliffe, N. A. (1982). The segregation and elimination of radio-fluorescent-labeled marine bacteria from the haemolymph of the shore crab, *Carcinus maenas*. *J. Mar. Biol. Assoc. UK* **62**, 819-833.
White, K. N., Ratcliffe, N. A. and Rossa, M. (1985). The antibacterial activity of haemocyte clumps in the gills of the shore crab, *Carcinus maenas*. *J. Mar. Biol. Assoc. UK* **65**, 857-870.
Winder, W. W. and Hardie, D. G. (1999). AMP-activated protein kinase, a metabolic master switch: possible roles in Type 2 diabetes. *Am. J. Physiol. Endocrinol. Metab.* **277**, E1-10.
Wolcott, T. G. and Hines, A. H. (1989). Ultrasonic biotelemetry of muscle activity from free-ranging marine animals: a new method for studying foraging by blue crabs (*Callinectes sapidus*). *Biol. Bull.* **176**, 50-56.
Wood, C. M. and Randall, D. J. (1981). Haemolymph gas transport, acid-base regulation, and anaerobic metabolism during exercise in the land crab *Cardisoma carnifex*. *J. Exp. Zool.* **218**, 23-35.