The Journal of Experimental Biology **198**, 349–358 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

A COMPARISON OF THE GILL PHYSIOLOGY OF TWO EURYHALINE CRAB SPECIES, *CALLINECTES SAPIDUS* AND *CALLINECTES SIMILIS*: ENERGY PRODUCTION, TRANSPORT-RELATED ENZYMES AND OSMOREGULATION AS A FUNCTION OF ACCLIMATION SALINITY

SABINE C. PILLER[1,*], RAYMOND P. HENRY[2], JEANNETTE E. DOELLER[1] AND DAVID W. KRAUS[1,†]

[1]*Department of Biology, University of Alabama at Birmingham, 1300 University Boulevard, Birmingham, AL 35294-1170, USA* and [2]*Department of Zoology, Auburn University, Auburn, AL 36849, USA*

Accepted 7 September 1994

Summary

***Callinectes sapidus* and *C. similis* co-occur in estuarine waters above 15‰ salinity. *Callinectes sapidus* also inhabits more dilute waters, but *C. similis* is rarely found below 15‰. Previous work suggests that *C. sapidus* may be a better hyperosmoregulator than *C. similis*. In this study, energy metabolism and the levels of transport-related enzymes in excised gills were used as indicators of adaptation to low salinity. Oxygen consumption rates and mitochondrial cytochrome content of excised gills increased in both species as acclimation salinity decreased, but to a significantly greater extent in *C. similis* gills. In addition, *C. similis* gills showed the same levels of carbonic anhydrase and Na^+/K^+-ATPase activities and the same degree of enzyme induction during low-salinity adaptation as has been reported for *C. sapidus* gills. However, hemolymph osmolality and ion concentrations were consistently lower in *C. similis* at low salinity than in *C. sapidus*. Therefore, although gills from low-salinity-acclimated *C. similis* have a higher oxygen consumption rate and more mitochondrial cytochromes than *C. sapidus* gills and the same level of transport-related enzymes, *C. similis* cannot homeostatically regulate their hemolymph to the same extent as *C. sapidus*.**

Key words: gills, mitochondria, crabs, salinity, carbonic anhydrase, Na^+/K^+-ATPase, hemolymph osmolality, spectrophotometry, respirometry.

Introduction

The greater blue crab *Callinectes sapidus* and the lesser blue crab *Callinectes similis* are closely related species of swimming crabs that co-occur in estuaries of the eastern and southern coasts of the United States (VanDen Avyle and Fowler, 1984; Millikin and Williams, 1984; Hsueh, 1992). The estuarine habitat is characterized by fluctuating salinities and temperatures, which can impose substantial ionic and osmotic stress on inhabitants. Although both *C. sapidus* and *C. similis* co-occur in estuarine waters from 30 to 15‰ salinity, *C. sapidus* also inhabits more dilute waters, as low as 0‰, and generally prefers low salinities (Perry, 1984; Hsueh *et al.* 1993). In contrast, *C. similis* is rarely found below 15‰ in Alabama estuaries (Hsueh *et al.* 1993). Similar salinity-limited distributions have been observed for these and other *Callinectes* species (Tagatz, 1967; Norse, 1978; Paul, 1982). Diet and habitat characteristics other than salinity were similar in both *C. sapidus* and *C. similis* in Alabama estuaries (Hsueh, 1992), suggesting that the difference in distribution may be related to salinity.

Both *C. sapidus* and *C. similis* are hyperosmoregulators, able to respond to fluctuating salinities by maintaining a hyperosmotic hemolymph concentration in low-salinity waters (King, 1965; Neff and Anderson, 1977; Mantel and Farmer, 1983; Mangum *et al.* 1985). On the basis of measurements of hemolymph osmolarity, Engel (1977) concluded that *C. sapidus* is the better hyperosmoregulator at lower salinities because it maintained higher hemolymph concentrations of Na^+ and Cl^- than *C. similis*.

Hyperosmoregulators are known to maintain high hemolymph osmolarity by active ion pumping in the gills, from the more dilute surrounding sea water into the more concentrated hemolymph (Mantel and Farmer, 1983; Lucu, 1990). The physiological mechanism for active ion uptake involves the coordinated function of a number of membrane-

*Present address: Australian National University, Department of Physiology, John Curtin School of Medical Research, Canberra, ACT 2601, Australia.
†Author for correspondence.

associated ion pumps and other transport-related enzymes. The initial step is believed to be independent Na^+/H^+ and Cl^-/HCO_3^- exchange across the apical membrane of the gill (Mantel and Farmer, 1983; Péqueux and Gilles, 1988), and cytoplasmic carbonic anhydrase is believed to supply H^+ and HCO_3^- by the catalysed hydration of respiratory CO_2 (Henry, 1984, 1988*a*,*b*). The Na^+/K^+-ATPase, located basolaterally, is responsible for the active transport of sodium into the hemolymph (for a review, see Towle, 1984). This enzyme requires energy in the form of ATP, which is mainly produced during oxidative phosphorylation in the mitochondria. Exposure to low salinity causes increases in both Na^+/K^+-ATPase and carbonic anhydrase activities in posterior and, to some extent, anterior gills (Towle *et al.* 1976; Neufeld *et al.* 1980; Henry and Cameron, 1982; Savage and Robinson, 1983; Henry, 1988*a*). The gill oxygen consumption rate also increases (King, 1965; Engel and Eggert, 1974; Mantel and Farmer, 1983; Péqueux and Gilles, 1988), indicating changes in both energy production and consumption of the gills, as well as possible amino acid oxidation.

In the present study, we ask whether differences in the energy metabolism and transport-related enzymes of *C. sapidus* and *C. similis* gills account for their observed salinity-dependent distribution. To answer this, functional mitochondrial characteristics of excised intact anterior and posterior gills were measured as oxygen consumption rate and cytochrome content. Because an increase in oxygen consumption rate may be accompanied by a greater dependence on ambient partial pressure of oxygen (Herreid, 1980), we measured the partial pressure of oxygen at which oxygen consumption rate is half-maximal and cytochromes are 50 % reduced. Activities of carbonic anhydrase and Na^+/K^+-ATPase in excised gills were also measured as well as hemolymph osmolality and ion content. All variables were measured as a function of salinity.

Materials and methods

Animal collection and maintenance

Callinectes sapidus (Rathbun) and *C. similis* (Williams) were collected between September 1991 and November 1992 from estuaries of the Gulf of Mexico near Dauphin Island, at the mouth of Mobile Bay, Alabama. Specimens were captured either offshore with an Otter Trawl from the research vessel *A. E. Verrill* or inshore with hand nets. During summer months, *C. sapidus* were also obtained from fish markets in Birmingham, Alabama. Within 10 h of collection, specimens were placed in aquaria containing artificial sea water at one of four test salinities (30 ‰, 20 ‰, 10 ‰ and 5 ‰), obtained by mixing sea salts (Tropic Marin) with dechlorinated tap water. Test salinities were maintained within ±2 ‰. Three to four animals were housed in each aquarium equipped with underground gravel filters. Animals were fed chopped fish or shrimp every second day. Ammonia and nitrate levels, checked with aquaria test kits (Tetra), remained below 15 μmol l^{-1} (0.25 mg l^{-1}) and 7 μmol l^{-1} (0.33 mg l^{-1}), respectively. Animals were acclimated to test salinities at 20±2 °C under a 14 h:10 h light:dark photoperiod for a minimum of 2 weeks prior to use in experiments. No *C. similis* survived the acclimation period in 5 ‰ salinity, even if subjected to gradual dilution of salinity. Experiments were performed on adult male and female intermolt animals with an average carapace width of 13 cm for *C. sapidus* and 8 cm for *C. similis*. All experiments were performed at 20 °C.

Gill preparation

Crabs were chilled on ice for 5–10 min to reduce activity prior to dissection. A 3 ml hemolymph sample was taken by syringe at the base of the first pereiopod and stored at −60 °C for later analysis of osmolality and ion content. All pereiopods were then removed, the carapace opened, and anterior gill pair 4 and posterior gill pair 8 were excised and placed in Millipore-filtered (0.45 μm) acclimation sea water at room temperature. Within 30 min of dissection, excised gills were mounted on specially designed gill holders and placed in the respirometer or spectrophotometer cuvette. Gill pairs 4 and 8, which are similar in size, were selected as representatives of anterior and posterior gills, respectively, for respirometric and spectrophotometric experiments, in which similar gill size was considered desirable for uniform solution stirring and to reduce variability associated with light pathlength for optical absorbance measurements. Gill pairs 3 and 7, which are different in size from each other, were excised and stored at −60 °C for later analysis of transport-related enzyme activity.

Gill holder

A gill holder was constructed with 60 mesh type 304 stainless-steel wire cloth (Small Parts, Miami, FL; Fig. 1). Its U shape was 14.5 mm wide at the base, with 11 mm long upright arms. In the cutout portion, two wires approximately 3 mm apart extended from the base to the top of the arms. A midsection of the gill, approximately 15 mm long, was held in a vertical orientation by sliding the afferent and efferent vessels onto these two upright wires. A short length of thin-walled silastic tubing placed around each arm secured the holder inside 16 mm diameter cuvettes. The holder was positioned in the spectrophotometer so that the sample light beam passed through the center of the gill (Fig. 1). The light beam pathlength through the gills, estimated from the measured thickness at the midsection, averaged 5.6 mm for the anterior gills and 7.7 mm for the posterior gills for *C. sapidus* and 4.0 mm for the anterior gills and 5.6 mm for the posterior gills for *C. similis*. The gill holder was positioned above the magnetic stirring bar in the respirometer.

Respirometry

A closed-chamber temperature-controlled polarographic respirometer, equipped with dual Pyrex chambers, titanium stoppers and magnetic stirring bars (Oxygraph 67097, Cyclobios-Paar, Graz, Austria), was used to measure the rates of oxygen consumption of excised gills. In the respirometer chamber, gills on the gill holder were bathed in Millipore-

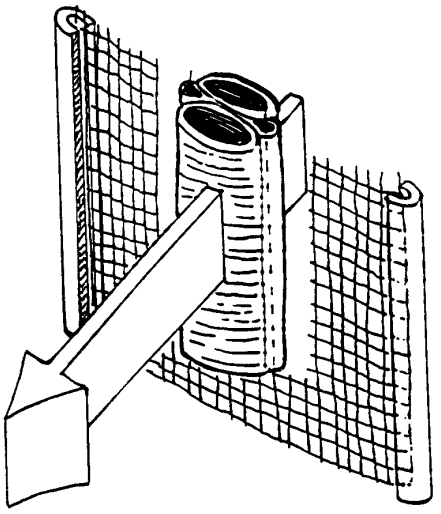

Fig. 1. Sketch of the gill holder with gill. The arrow indicates the light beam passing through the tissue in the spectrophotometric experiments.

filtered (0.45 μm) acclimation sea water containing 5 mmol l^{-1} L-alanine as substrate (Pressley and Graves, 1983). Oxygen consumption rates were calculated as the slope of the declining P_{O_2} recording at specific P_{O_2} values between 70 and 90 % air saturation, well above the P_{O_2} at which oxygen consumption rates of all gills began to show conformity, between 24 and 48 % air saturation, irrespective of acclimation salinity (data not shown). Blank oxygen consumption rates were determined before and after gills were added to the chamber. Gill mass (60–150 mg wet mass, 5–20 mg dry mass) and chamber volume (5 ml) were similar in both respirometer and spectrophotometer cuvettes (see below). Oxygen was completely consumed within 2–3 h in the closed chamber. Normoxic oxygen consumption rate remained constant for at least 8 h after excision. Gill dry mass was used to normalize oxygen consumption rate, $\dot{M}_{O_2}$, and is reported as pmol O_2 s^{-1} mg^{-1} dry mass.

Optical spectrophotometry

Optical spectra were acquired with a recording spectrophotometer (Cary 14) equipped with a scattered transmission accessory and a digital data-acquisition and analysis system (Aviv Associates, Lakewood, NJ). The spectrophotometer was modified to record optical spectra while tissues were held under specified gas mixtures (Wittenberg and Wittenberg, 1985, 1987). Excised gills on the holder were placed in the temperature-controlled sample cuvette containing 3.5–5 ml of stirred acclimation sea water with 5 mmol l^{-1} L-alanine. A piece of white transparent plastic in acclimation sea water with 5 mmol l^{-1} L-alanine was used to scatter the light beam in the reference cuvette. Humidified gas mixtures of air and nitrogen ranging from 100 % air to 0 % air, prepared with a mass flow controller (Tylan, Carson, CA), flowed through the 3 ml gas space above the liquid at a rate of 100 ml min^{-1}. During changes in gas tension, optical density was continuously recorded at 450 nm, which is close to the absorbance maximum of reduced cytochrome aa_3. Kinetic events were complete within 15 min, after which optical absorbance spectra were recorded over a range of values from 650 to 400 nm at 1 nm intervals. Difference spectra were constructed from the results obtained from gills in sea water equilibrated with less than 100 % air minus the results from gills in air-equilibrated sea water. The relative reduction of cytochromes at specific P_{O_2} values was calculated using optical density differences (ΔOD) at 445 nm and 470 nm for cytochrome aa_3, 550 nm and 540 nm for cytochrome *c* and 560 nm and 580 nm for cytochrome *b* in difference spectra. The ΔOD of cytochrome aa_3 in difference spectra of reduced minus oxidized gills was also used as a measure of cytochrome content.

Isolation of gill mitochondria

Mitochondria were isolated from gills of *C. sapidus* following the procedure of King (1966). Briefly, gills were excised and homogenized on ice with a mortar and pestle. Shearing was increased by adding clean sea sand. Sheared tissue was suspended in 0.05 mol l^{-1} potassium-phosphate-buffered 0.85 mol l^{-1} sucrose (pH 7.4) in a 1:10 (w:v) mixture. The suspension was centrifuged at 2500 ***g*** for 10 min and the supernatant was centrifuged at 18 000 ***g*** for 50 min at 4 °C (Sorvall RC2-B). The mitochondrial pellet was resuspended in 2 volumes of sucrose buffer and used in the spectrophotometer. Spectral signatures of the cytochromes were obtained by constructing difference spectra of mitochondria reduced with dithionite minus mitochondria oxidized in air.

Enzyme assays

Gill pairs 3 and 7 of *C. similis* were thawed to 0 °C on ice and cut in half for use in both the carbonic anhydrase and Na^+/K^+-ATPase assays. For the carbonic anhydrase assay, gills were homogenized in 5 volumes of cold buffer containing 225 mmol l^{-1} mannitol, 75 mmol l^{-1} sucrose and 10 mmol l^{-1} Tris (pH 7.4). Homogenates were sonicated at 25 W for 30 s (Heat Systems Ultrasonics) and centrifuged at 12 000 ***g*** for 20 min at 4 °C (Sorvall RC-5B). The catalysed rate of CO_2 hydration was determined electrometrically using the delta pH method described by Henry (1991). Protein concentration was determined by Coomassie Blue (BioRad Laboratories), and carbonic anhydrase activity was reported as μmol CO_2 mg^{-1} protein min^{-1}.

For the Na^+/K^+-ATPase assay, *C. similis* gills were placed in 10 volumes of cold buffer containing 250 mmol l^{-1} sucrose, 50 mmol l^{-1} imidazole, 2 mmol l^{-1} ETDA, 5 mmol l^{-1} mercaptoethanol and 0.1 % deoxycholate (pH 7.4), and homogenized in a motor-driven Teflon–glass homogenizer. Homogenates were sonicated as above and centrifuged at 8500 ***g*** for 20 min at 4 °C (Sorvall RC-5B). Activity was determined from the rate of oxidation of NADH using a pyruvate kinase/lactate dehydrogenase-linked assay (Gibbs

and Somero, 1989). Protein concentrations were measured as above, and activity was reported as nmol ATP mg^{-1} protein min^{-1}.

Hemolymph osmolality and ion content

Hemolymph and seawater osmolality were determined using a vapor pressure osmometer (Wescor 5100C). Chloride concentrations were measured with a chloridometer (Buchler–Cotlove). Hemolymph and seawater sodium and potassium concentrations were determined using a flame photometer (Radiometer FLM3). Measurements were obtained using previously frozen hemolymph after samples had been thawed at room temperature, then sonicated and centrifuged to remove clots.

Data analysis

Data are presented in the text and shown in figures as mean ± standard deviation (S.D., error bars in figures), with the number of repetitions in parentheses. Statistical comparisons were made with Student's *t*-test and analysis of covariance (ANCOVA). Statistical significance was assigned at $P<0.05$ (marked by asterisks in figures).

Results

Oxygen consumption rate as a function of salinity

Normoxic oxygen consumption rates of excised gills acclimated to 30‰ salinity were 11.7±4.6 (4) for anterior gills and 10.4±4.1 (4) pmol O_2 s^{-1} mg^{-1} dry mass for posterior gills from *C. sapidus* and 11.5±2.8 (6) and 13.8±2.0 (6) pmol O_2 s^{-1} mg^{-1} dry mass from *C. similis*. These rates compare well with others reported for *C. sapidus* gills (Engel and Eggert, 1974; Cantelmo and Rao, 1978).

In both species, the oxygen consumption rate of gills was nearly the same at 30‰ and increased as acclimation salinity decreased (Fig. 2). However, at each salinity tested except 30‰, the anterior and posterior gills of *C. similis* generally had significantly higher oxygen consumption rates than those of *C. sapidus*. The rise in oxygen consumption rate as acclimation salinity decreased (slope of fitted lines in Fig. 2) was also significantly greater, approximately 3.1-fold, in gills of *C. similis* than in those of *C. sapidus*. The oxygen consumption rates of gills from 10‰ salinity-acclimated *C. sapidus* were 20% (anterior gills) and 33% (posterior gills) higher than those of gills from 30‰ salinity-acclimated animals. In contrast, oxygen consumption rates of gills from 10‰ salinity-acclimated *C. similis* were 103% (anterior gill) and 75% (posterior gill) higher those that of gills from 30‰ salinity-acclimated animals.

Cytochrome content of gills as a function of salinity

Although very few features were observable in the direct optical spectra of excised gills, mathematically constructed difference spectra exhibited abundant and pronounced features. Difference spectra of gills of each species in nitrogen-equilibrated sea water minus gills in air-equilibrated sea water

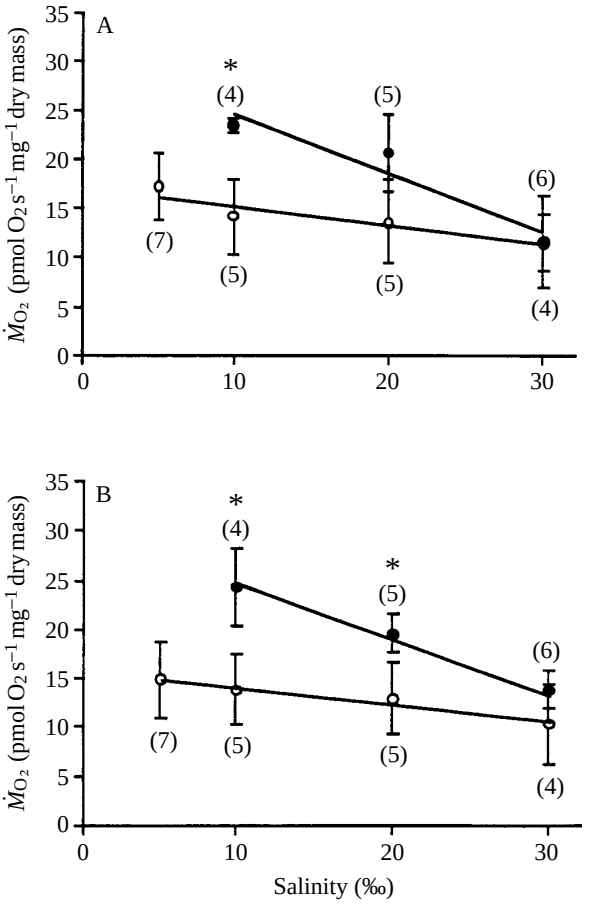

Fig. 2. Oxygen consumption rate, $\dot{M}_{O_2}$, of gills from *Callinectes similis* (filled circles) and *C. sapidus* (open circles) as a function of salinity. (A) Anterior gills 4. (B) Posterior gills 8. In this and later figures, values are means ± S.D.; *N* values are given in parentheses. Asterisks mark statistically significant differences between the species.

were similar to difference spectra of reduced minus oxidized isolated mitochondria from *C. sapidus* gills (Fig. 3). Both spectra exhibited wavelength maxima typical of reduced minus oxidized mitochondrial cytochromes: cytochrome aa_3 at 603 nm and 445 nm, cytochrome *c* at 550 nm and cytochrome *b* at 557 nm and 426 nm (Fig. 3). These wavelength maxima compare well with those in optical spectra from *C. sapidus* claw muscles (Gilles, 1973).

The ΔOD of each cytochrome in difference spectra increased as a function of decreasing acclimation salinity in anterior and posterior gills of both species, as represented by the increase in the ΔOD of cytochrome aa_3 (Fig. 4). However, cytochrome content was higher in *C. similis* gills than in *C. sapidus* gills at all salinities tested. The increase in cytochrome aa_3 content from high to low acclimation salinity (slope of fitted lines in Fig. 4) was 2.3 times greater in anterior gills and four times greater in posterior gills of *C. similis* compared with the corresponding gills of *C. sapidus*. The cytochrome aa_3 contents of gills from 10‰ salinity-acclimated *C. sapidus* were 136% (anterior gill) and 71% (posterior gill) higher than

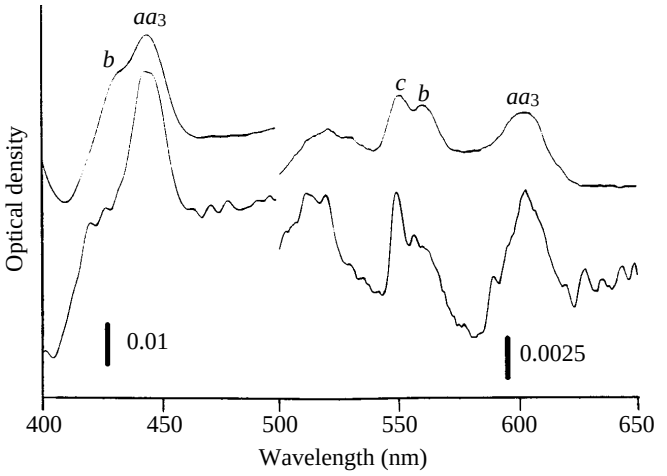

Fig. 3. Difference spectra of anterior gill 4 from *Callinectes similis* acclimated to 30‰ salinity in nitrogen-equilibrated sea water minus the same gill in air-equilibrated sea water (lower trace) and isolated *C. sapidus* gill mitochondria in buffer containing dithionite minus the same mitochondria in air-equilibrated buffer (upper trace). Wavelength maxima of cytochromes aa_3, *b* and *c* are given in the text. This gill difference spectrum is representative of the gill spectra used in this study. Scales are expressed in units of optical density.

those of gills from 30‰ salinity-acclimated animals. The cytochrome aa_3 contents of gills from 10‰ salinity-acclimated *C. similis* were 103% (anterior gills) and 75% (posterior gills) higher than those of gills from 30‰ salinity-acclimated animals.

Oxidation/reduction state of mitochondrial cytochromes as a function of P_{O_2}

Difference spectra of gills in nitrogen-equilibrated sea water minus gills in air-equilibrated sea water were used to determine the maximum cytochrome reduction achieved under these conditions, calculated as maximum ΔOD. The relative reduction of individual mitochondrial cytochromes at each ambient P_{O_2} was calculated as the ratio of ΔOD taken from difference spectra of gills in sea water at that P_{O_2} minus gills in air-equilibrated sea water to maximum ΔOD. The ambient P_{O_2} resulting in a 50% reduction of cytochrome aa_3, calculated using the Hill equation and called $P(\mathrm{cyt})_{50}$, did not significantly change in anterior and posterior gills of both species as acclimation salinity was decreased (Fig. 5). The $P(\mathrm{cyt})_{50}$ of cytochromes *b* and *c* followed the same pattern (data not shown).

Oxygen consumption rate as a function of P_{O_2}

The ambient P_{O_2} at which oxygen consumption rate was half-maximal, calculated using the Hill equation and called $P(\dot{M}_{O_2})_{50}$, did not change significantly in gills of both species at different acclimation salinities (Fig. 6).

Transport-related enzyme activity as a function of salinity

At 30‰ salinity, carbonic anhydrase activity of *C. similis* gills was relatively low and not significantly different between

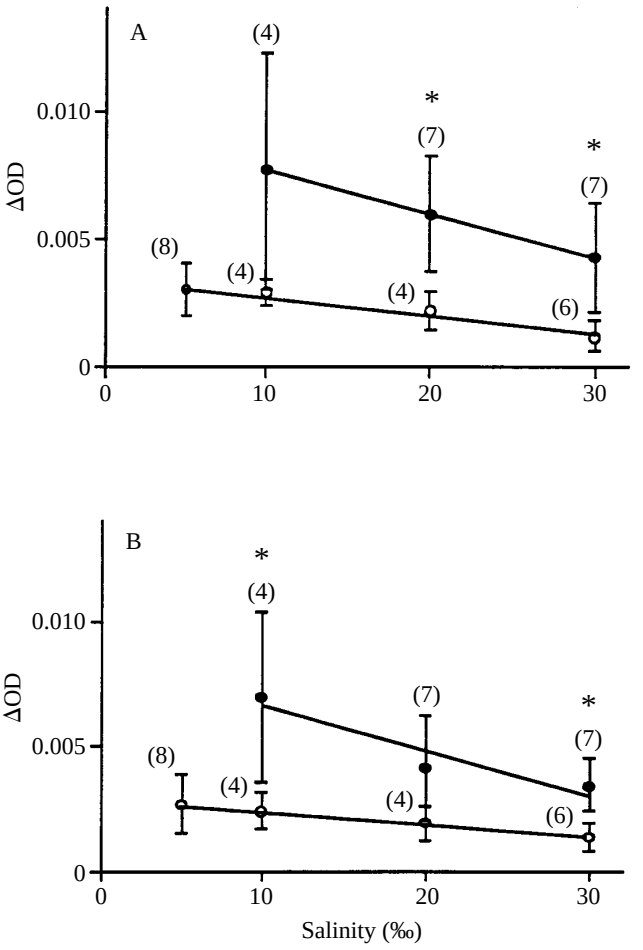

Fig. 4. Difference in optical density (ΔOD) of cytochrome aa_3 at 445 nm minus 470 nm of gills from *Callinectes similis* (filled circles) and *C. sapidus* (open circles) as a function of salinity. (A) Anterior gills 4. (B) Posterior gills 8. Optical density was normalized for the light pathlength.

anterior and posterior gills (Fig. 7). Upon acclimation to low salinity, carbonic anhydrase activity increased predominantly in the posterior gills, with an approximately ninefold increase in posterior gill 7 at 10‰ salinity (Fig. 7). These results are virtually identical to those seen in *C. sapidus* anterior and posterior gills at 28‰ and 10‰ salinity, respectively (Henry and Cameron, 1982; Henry, 1988*a*). Carbonic anhydrase activity also increased significantly (doubled) in *C. similis* anterior gill 3 at 10‰ salinity, a result not seen in *C. sapidus* anterior gills (Henry and Cameron, 1982).

The Na^+/K^+-ATPase activity of *C. similis* posterior gills increased by fourfold betwen 30‰ and 10‰ salinity (Fig. 7). Values for Na^+/K^+-ATPase activity in Fig. 7 are within the range previously reported for *C. sapidus* gills (100–400 nmol ATP mg^{-1} protein min^{-1}; Neufeld *et al.* 1980) and show the same magnitude increase as the posterior gills of *C. sapidus* (two- to fourfold; Towle *et al.* 1976; Neufeld *et al.* 1980) and other euryhaline crustaceans (Siebers *et al.* 1982; Holliday, 1985).

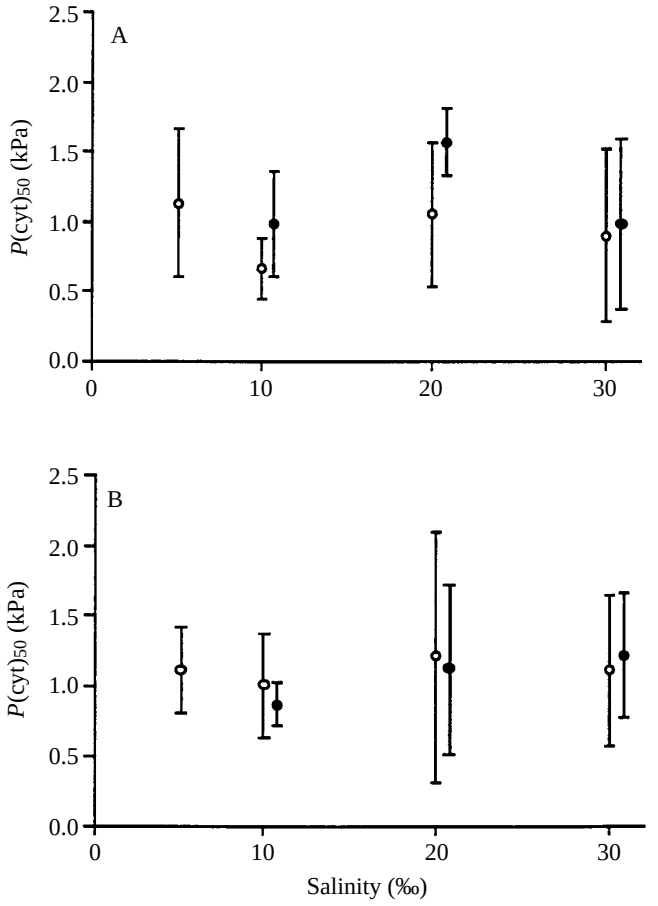

Fig. 5. Partial pressure of oxygen where cytochrome aa_3 is 50 % reduced, $P(\mathrm{cyt})_{50}$, of gills from *Callinectes similis* (filled circles) and *C. sapidus* (open circles) as a function of salinity. (A) Anterior gills 4. (B) Posterior gills 8. Each data point represents an average of 4–8 experiments. Data for *C. similis* have been offset by 0.8‰ for clarity.

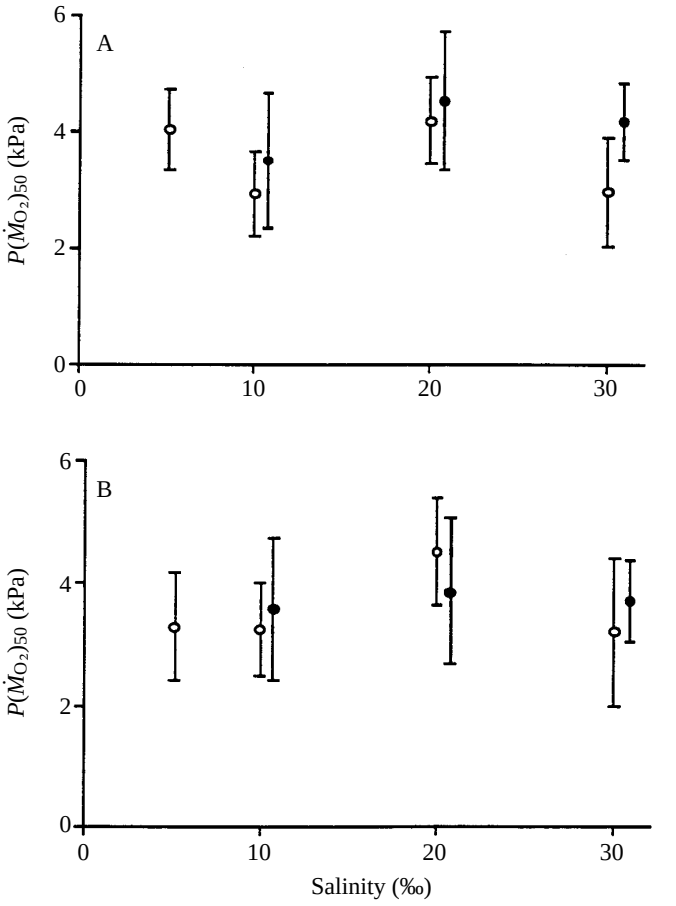

Fig. 6. Partial pressure of oxygen where oxygen consumption rate is half-maximal, $P(\dot{M}_{O_2})_{50}$, of gills from *Callinectes similis* (filled circles) and *C. sapidus* (open circles) as a function of salinity. (A) Anterior gills 4. (B) Posterior gills 8. Each data point represents an average of 4–7 experiments. Data for *C. similis* have been offset by 0.8 ‰ for clarity.

Hemolymph osmolality and ion content as a function of salinity

Hemolymph osmolality of both species acclimated to 30 ‰ salinity was nearly equal to that of the acclimation sea water, indicating osmoconformity, whereas hemolymph osmolality of animals acclimated to lower salinities was consistently above acclimation seawater values (Fig. 8), indicating hyperosmoregulation at lower salinities. Concentrations of Na^+, Cl^- and K^+ in the hemolymph of both species acclimated to 30 ‰ salinity were consistently below those of the acclimation sea water, indicating ionoregulation, whereas ion concentrations in the hemolymph of animals acclimated to salinities below 20 ‰ were consistently above acclimation seawater values, also indicating ionoregulation (Fig. 8).

Callinectes sapidus maintained a significantly higher hemolymph osmolality at 10 ‰ salinity than did *C. similis* (Fig. 8A). In addition, Na^+, Cl^- and K^+ were maintained at higher hemolymph concentrations in *C. sapidus* acclimated to 10 ‰ salinity than in *C. similis* (Fig. 8B–D).

Discussion

Crustacean gills are situated at an interface between the animal and its environment and are important in respiration, acid–base balance and osmotic and ionic regulation (Henry and Cameron, 1983; Henry, 1987; Lucu, 1990). Most euryhaline crab species are hyperosmoregulators at low salinities, actively pumping ions from the sea water into the hemolymph (Mantel and Farmer, 1983; Lucu, 1990). The two main enzymes involved in the ion-pumping process are the ouabain-sensitive Na^+/K^+-ATPase (Towle, 1984; Burnett and Towle, 1990) and carbonic anhydrase (Henry, 1984, 1988*a*). The responses of euryhaline crab species acclimated to low salinity include increased oxygen consumption rate of whole animals (Sabourin, 1983) and excised gills (Engel and Eggert, 1974; Engel *et al.* 1975; Péqueux and Gilles, 1988), proliferation of mitochondria-rich chloride cells, with changes in mitochondrial ultrastructure (Copeland and Fitzjarrell, 1968; Péqueux and Gilles, 1988), increased gill Na^+/K^+-ATPase activity (Towle *et al.* 1976; Neufeld *et al.* 1980; Savage and Robinson, 1983; Péqueux and Gilles, 1984), increased gill

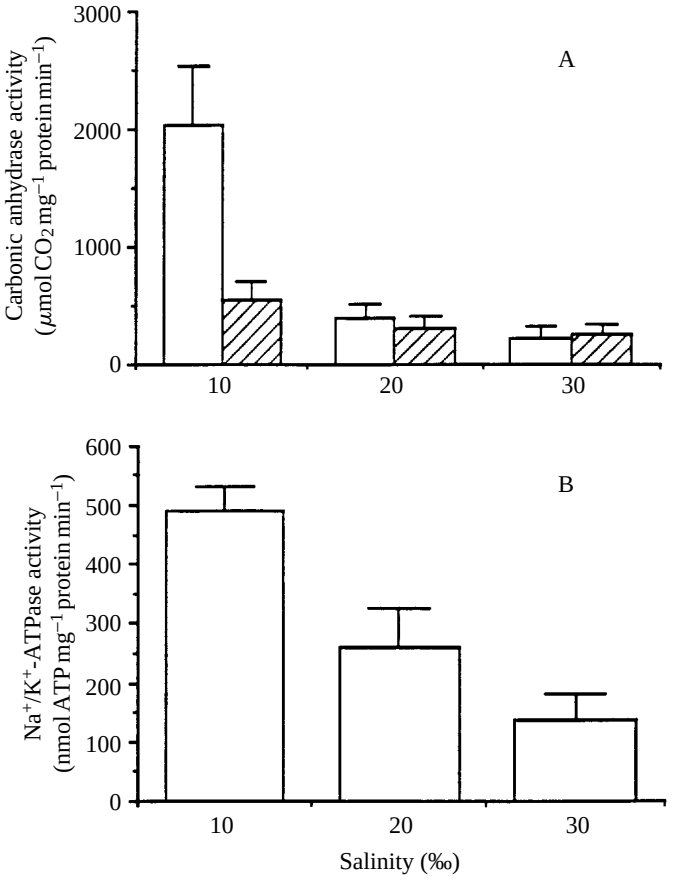

Fig. 7. Activities of transport-related enzymes in *Callinectes similis* anterior gills 3 (hatched bars) and posterior gills 7 (open bars) as a function of salinity. (A) Carbonic anhydrase. (B) Na^+/K^+-ATPase. Values are means + S.D., each data point represents an average of 4–6 experiments.

carbonic anhydrase activity (Henry and Cameron, 1982; Henry, 1984, 1988*b*), decreased water and ion permeability of whole animals (Cantelmo, 1977; Oglesby, 1981) and of isolated tissue (Mantel and Farmer, 1983; Péqueux and Gilles, 1988), increased amino acid oxidation (Pierce, 1982; Gilles, 1983; Pressley and Graves, 1983), an increase in gill membrane phospholipids (Whitney, 1974) and a decrease in gill ATP content (Engel *et al.* 1975). These studies indicate that low-salinity stress induces a suite of changes in order to maintain osmotic and ionic homeostasis.

Gills of both *C. sapidus* and *C. similis* showed increased oxygen consumption rates with decreasing acclimation salinity, as has been shown for many osmoregulating animals (Mantel and Farmer, 1983). However, *C. similis* gills had a significantly higher oxygen consumption rate than *C. sapidus* gills at lower salinity. Increases in both amino acid oxidation and Na^+/K^+-ATPase activity have been proposed to account for part of the increased oxygen consumption rate at low salinity (Gilles, 1983; Mantel and Farmer, 1983; Pressley and Graves, 1983). It is noteworthy that at 30 ‰ salinity, where osmotic stress may be minimal for each species, gill oxygen consumption rates were not significantly different. However, each crab species appears to be ionoregulating at both higher and lower salinities (Fig. 8; Engel, 1977). The maintenance of ion gradients in either direction may involve active transport and any change in salinity may stimulate oxygen consumption.

In parallel with increased oxygen consumption rates, the mitochondrial cytochrome content in anterior and posterior gills of both *C. sapidus* and *C. similis* increased as acclimation salinity decreased, possibly reflecting an increase in the number of mitochondria present, as has been demonstrated ultrastructurally (Copeland and Fitzjarrell, 1968). At low salinity, the higher oxygen consumption rate and mitochondrial cytochrome content of *C. similis* gills compared with those of *C. sapidus* gills suggest that *C. similis* gills have a significantly greater capacity for mitochondrial ATP production at low salinity.

A rise in the ATP demand of ion-transporting tissue results in an elevated electron flux through the mitochondrial electron transport chain and a higher oxygen consumption rate (Whittam, 1963; Péqueux and Gilles, 1984). Crucial to these processes is a continuous supply of oxygen, and if oxygen diffusion becomes limited, oxidative phosphorylation rates decline proportionally. An increased tissue oxygen consumption rate causes an increase in the P_{O_2} at which diffusion becomes limited, called the critical P_{O_2} (Herreid, 1980), as well as an increase in the $P(\dot{M}_{O_2})_{50}$ and possibly in the $P(\mathrm{cyt})_{50}$. Although the gills of *C. similis* and *C. sapidus* showed a significant increase in oxygen consumption rate at dilute salinities, both $P(\dot{M}_{O_2})_{50}$ and $P(\mathrm{cyt})_{50}$ remained constant in gills of both species at all salinities tested. This lack of change in $P(\dot{M}_{O_2})_{50}$ and $P(\mathrm{cyt})_{50}$ may occur if the average diffusion distance in low-salinity-acclimated gills remains constant and the additional mitochondria maintain an oxygen consumption rate comparable to that of the few mitochondria in high-salinity-acclimated gills (see Rumsey *et al.* 1990). Alternatively, the diffusion distance to mitochondria may be reduced and oxygen diffusion enhanced if gills of *Callinectes* species show a great increase in membrane infoldings, similar to those found in the gills of *Eriocheir sinensis* acclimated to low salinity (Péqueux and Gilles, 1988). Enhanced oxygen diffusion would enable an increased oxygen consumption rate without an accompanying rise in critical P_{O_2}, $P(\dot{M}_{O_2})_{50}$ or $P(\mathrm{cyt})_{50}$ (Herreid, 1980). Assuming that the ultrastructural changes at low salinity acclimation observed in *C. sapidus* gills (Copeland and Fitzjarrell, 1968) support elevated energy metabolism without altering $P(\dot{M}_{O_2})_{50}$ and $P(\mathrm{cyt})_{50}$, our data suggest that homologous changes in *C. similis* gills are perhaps more pronounced.

In most euryhaline species, the larger posterior gills 6 and 7 are major sites of ion transport, containing higher activities of Na^+/K^+-ATPase and carbonic anhydrase and more mitochondria-rich ion-pumping cells than the anterior and other posterior gills (Neufeld *et al.* 1980; Péqueux *et al.* 1984; Henry, 1987; Lucu, 1990). Gills 6 and 7 also show the largest increase in Na^+/K^+-ATPase activity in response to low-salinity acclimation (Neufeld *et al.* 1980). However, Na^+/K^+-ATPase activity in gill pairs 4, 5 and 8 also increases significantly in

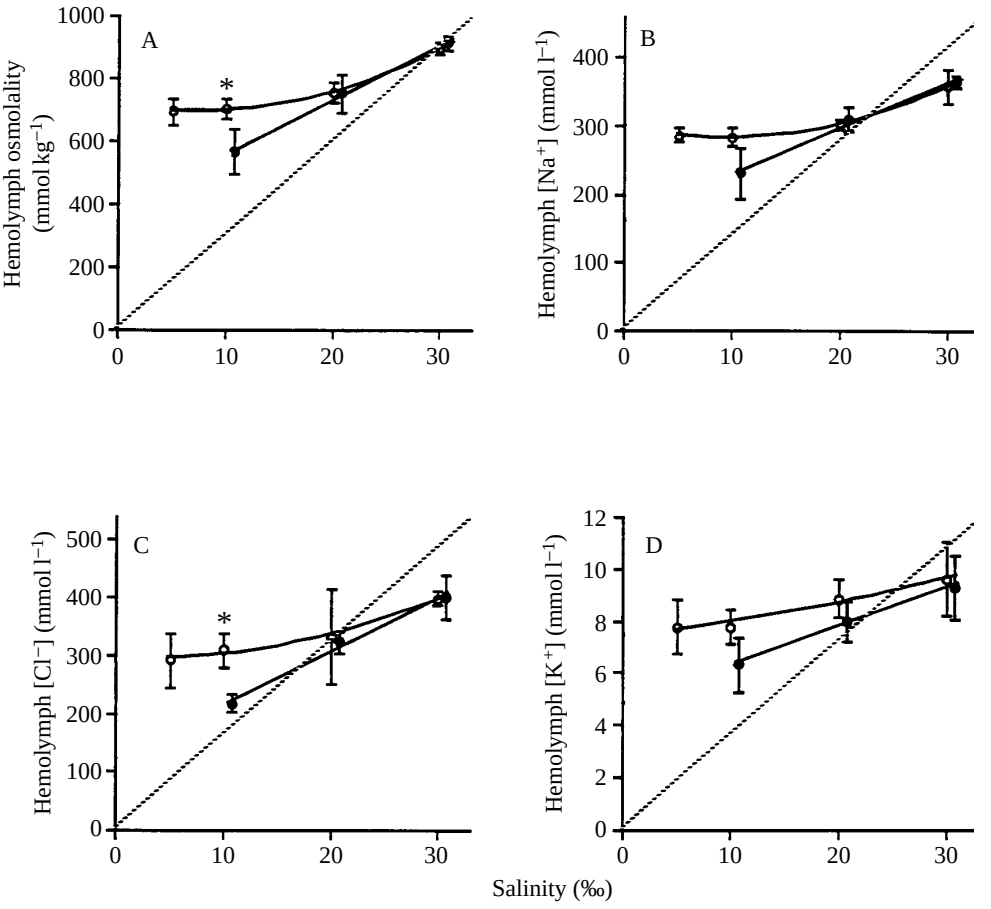

Fig. 8. Hemolymph osmolality and ion concentrations for *Callinectes similis* (filled circles) and *C. sapidus* (open circles) as a function of salinity. Data for *C. similis* have been offset by 0.8‰ for clarity. the dashed line in each graph is the line of equality. Data were best fitted with a non-linear regression for *C. sapidus* and a linear regression for *C. similis*. Values for r^2 were greater than 0.97 for all regressions. (A) Osmolality. (B) Sodium concentration. (C) Chloride concentration. (D) Potassium concentration.

response to low-salinity acclimation (Neufeld *et al.* 1980), indicating that ion-pumping activity occurs in these gills as well. In the present study, no significant differences in oxygen consumption rate and mitochondrial cytochrome content were observed between anterior gill 4 and posterior gill 8 in both *C. sapidus* and *C. similis*. The similar capacity of both gills 4 and 8 to undergo salinity-dependent ion-pumping adaptation, albeit to a lesser extent than that in gills 6 and 7, may contribute to this similarity in observed responses. These results are in agreement with the respirometric measurements made by Engel *et al.* (1975), in which excised *C. sapidus* gills 1–5 were grouped as anterior and 6–8 were grouped as posterior. In their study, no consistent differences in oxygen consumption rates were found between anterior and posterior gills (Engel *et al.* 1975). In all excised gills, oxygen consumption rates at low salinity cannot be ascribed solely to higher ion-pumping rates, but may also reflect other cellular adjustments, such as increased amino acid oxidation for cell volume regulation (for review see Gilles, 1983).

At low salinity, *C. similis* is a weaker osmotic and ionic regulator than is *C. sapidus* (Engel, 1977; Fig. 7). Osmotic and ionic regulation results from the interaction between active uptake of ions from the medium and the outward diffusion of ions across the gills and loss by bulk urine flow (e.g. Cameron, 1978). In the present study, mitochondrial ATP production appears to be greater in *C. similis* gills at low salinity than in *C. sapidus* gills. The magnitude of activity of the two major enzymes involved in the uptake process, Na^+/K^+-ATPase and carbonic anhydrase, and their degree of induction in low salinity are virtually identical in the two species, suggesting that both species have similar ion uptake capabilities. The difference between the two species may then lie in the diffusive loss of ions across the gills, with the gills of *C. similis* possibly being more permeable. Euryhaline marine organisms such as *C. sapidus* do not appear to reduce gill permeability in low salinity water (Cameron, 1978); rather, they compensate for ion loss by the activation of ion uptake mechanisms. If the gills of *C. similis* are more permeable than the gills of *C. sapidus* (S. C. Piller, J. E. Doeller and D. W. Kraus, in preparation), then energy metabolism and uptake mechanisms may not be able to compensate for diffusive ion loss below a certain critical salinity, and this may limit the degree to which *C. similis* can invade the more dilute waters of estuaries.

We wish to thank the crew of the research vessel *A. E. Verrill* and the staff at Dauphin Island Sea Laboratory, Alabama, for their help in animal collection and the Department of Biology, University of Alabama at Birmingham, for providing logistical and financial assistance. This work forms part of a thesis submitted by S.C.P. for the

Master of Science degree at UAB and was supported by grants from the Austrian Ministry of Science and Research and the Alabama Academy of Science to S.C.P., NSF IBN9304844 to R.P.H. and by NSF IBN9118048 to D.W.K. and J.E.D.

References

BURNETT, L. E. AND TOWLE, D. W. (1990). Sodium ion uptake by perfused gills of the blue crab *Callinectes sapidus*: effects of ouabain and amiloride. *J. exp. Biol.* **149**, 293–305.

CAMERON, J. N. (1978). NaCl balance in blue crabs, *Callinectes sapidus*, in fresh water. *J. comp. Physiol.* **123**, 127–135.

CANTELMO, A. C. (1977). Water permeability of isolated tissues from decapod crustaceans. I. Effect of osmotic conditions. *Comp. Biochem. Physiol.* **58**A, 343–348.

CANTELMO, A. C. AND RAO, K. R. (1978). The effects of pentachlorophenol (PCP) and 2,4-dinitrophenol (DNP) on the oxygen consumption of tissues from the blue crab, *Callinectes sapidus*, under different osmotic conditions. *Comp. Biochem. Physiol.* **60**C, 215–219.

COPELAND, D. E. AND FITZJARRELL, A. T. (1968). The salt absorbing cells in the gills of the blue crab (*Callinectes sapidus* Rathbun) with notes on modified mitochondria. *Z. Zellforsch. mikrosk. Anat.* **92**, 1–22.

ENGEL, D. W. (1977). Comparison of the osmoregulatory capabilities of two portunid crabs, *Callinectes sapidus* and *C. similis*. *Mar. Biol.* **41**, 275–279.

ENGEL, D. W. AND EGGERT, L. D. (1974). The effect of salinity and sex on the respiration rates of excised gills of the blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol.* **47**A, 1005–1011.

ENGEL, D. W., FERGUSON, R. L. AND EGGERT, L. D. (1975). Respiration rates and ATP concentrations in the excised gills of the blue crab as a function of salinity. *Comp. Biochem. Physiol.* **52**A, 669–673.

GIBBS, A. AND SOMERO, G. (1989). Pressure adaptation of Na^+/K^+ATPase in gills of marine teleosts. *J. exp. Biol.* **143**, 475–492.

GILLES, R. (1973). Oxygen consumption as related to the amino-acid metabolism during osmoregulation in the blue crab *Callinectes sapidus*. *Neth. J. Sea Res.* **7**, 280–289.

GILLES, R. (1983). Review: Volume maintenance and regulation in animal cells: some features and trends. *Molec. Physiol.* **4**, 3–16.

HENRY, R. P. (1984). The role of carbonic anhydrase in blood ion and acid–base regulation. *Am. Zool.* **24**, 241–251.

HENRY, R. P. (1987). Membrane-associated carbonic anhydrase in gills of the blue crab, *Callinectes sapidus*. *Am. J. Physiol.* **252**, R966–R971.

HENRY, R. P. (1988*a*). The subcellular distribution of carbonic anhydrase activity in the gills of the blue crab, *Callinectes sapidus*. *J. exp. Zool.* **245**, 1–8.

HENRY, R. P. (1988*b*). Multiple functions of crustacean gill carbonic anhydrase. *J. exp. Zool.* **248**, 19–24.

HENRY, R. P. (1991). Techniques for measuring carbonic anhydrase activity *in vitro*: the electrometric delta pH and pH stat methods. In *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (ed. S. J. Dodgson, R. E. Tashien, G. Gros and N. D. Carter), pp. 119–125. New York: Plenum Press.

HENRY, R. P. AND CAMERON, J. N. (1982). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. *J. exp. Zool.* **221**, 309–323.

HENRY, R. P. AND CAMERON, J. N. (1983). The role of carbonic anhydrase in respiration, ion regulation and acid–base balance in the aquatic crab *Callinectes sapidus* and the terrestrial crab *Gecarcinus lateralis*. *J. exp. Biol.* **103**, 205–223.

HERREID, C. F. (1980). Hypoxia in invertebrates. *Comp. Biochem. Physiol.* **67**A, 311–320.

HOLLIDAY, C. W. (1985). Salinity-induced changes in gill Na,K-ATPase activity in the mud fiddler crab, *Uca pugnax*. *J. exp. Zool.* **233**, 199–208.

HSUEH, P. (1992). A comparative study of the population dynamics, life history characteristics and physiological ecology of *Callinectes similis* and *C. sapidus* in estuarine environments of the Northern Gulf of Mexico. Doctoral thesis, University of Alabama at Birmingham.

HSUEH, P., MCCLINTOCK, J. B. AND HOPKINS, T. S. (1993). Population dynamics and life history characteristics of the blue crabs *Callinectes similis* and *C. sapidus* in bay environments of the Northern Gulf of Mexico. *Mar. Ecol. PSZNI* (in press).

KING, E. N. (1965). The oxygen consumption of intact crabs and excised gills as a function of decreased salinity. *Comp. Biochem. Physiol.* **15**, 93–102.

KING, E. N. (1966). Oxidative activity of crab gill mitochondria as a function of osmotic concentration. *Comp. Biochem. Physiol.* **17**, 245–258.

LUCU, C. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. *Comp. Biochem. Physiol.* **97**A, 297–306.

MANGUM, C. P., MCMAHON, B. R., DEFUR, P. L. AND WHEATLY, M. G. (1985). Gas exchange, acid–base balance and the oxygen supply to tissue during a molt of the blue crab *Callinectes sapidus*. *J. Crust. Biol.* **5**, 188–206.

MANTEL, L. H. AND FARMER, L. L. (1983). Osmotic and ionic regulation. In *The Biology of Crustacea* (ed. in chief E. Bliss), vol. 5 (ed. L. H. Mantel), pp. 53–161. London: Academic Press.

MILLIKIN, M. R. AND WILLIAMS, A. B. (1984). Synopsis of biological data on the blue crab, *Callinectes sapidus* Rathbun. NOAA Tech. Rept. NMFS 1. *FAO Fisheries Synopsis* **138**, 1–39.

NEFF, J. M. AND ANDERSON, J. W. (1977). The effects of copper (II) on molting and growth of juvenile lesser blue crabs *Callinectes similis* Williams. In *Pollutant Effects on Marine Organisms* (ed. C. S. Giam), pp. 155–165. Toronto: Lexington Books.

NEUFELD, G. J., HOLLIDAY, C. W. AND PRITCHARD, J. B. (1980). Salinity adaptation of gill Na,K-ATPase in the blue crab, *Callinectes sapidus*. *J. exp. Zool.* **211**, 215–224.

NORSE, E. A. (1978). Physicochemical and biological stressors as distributional determinants of Caribbean and tropical Eastern Pacific swimming crabs. In *Energy and Environmental Stress in Aquatic Systems* (ed. J. H. Thorp and J. W. Gibbons), pp. 120–140. Technical Information Center, US Department of Energy.

OGLESBY, L. C. (1981). Volume regulation in aquatic invertebrates. *J. exp. Zool.* **215**, 289–301.

PAUL, R. K. G. (1982). Observations on the ecology and distribution of swimming crabs of the genus *Callinectes* (Decapoda, Brachyura, Portunidae) in the Gulf of California, Mexico. *Crustaceana* **42**, 96–100.

PÉQUEUX, A. AND GILLES, R. (1984). Control of the extracellular fluid osmolality in crustaceans. In *Osmoregulation in Estuarine and Marine Animals*. Proc 5th Conf. Eur. Soc. Comp. Physiol. Biochem., Taormina, Italy (ed. A. Péqueux, R. Gilles and L. Bolis), pp. 17–34. *Lecture Notes on Coastal and Estuarine Studies*, vol. 9. Berlin, Heidelberg, New York, Tokyo: Springer Verlag.

PÉQUEUX, A. AND GILLES, R. (1988). NaCl transport in gills and related structures. In *Comparative and Environmental Physiology*,

vol. 1, *NaCl Transport in Epithelia* (ed. R. Greger), pp. 2–73. Berlin: Springer Verlag.

PÉQUEUX, A., MARCHAL, A., WANSON, S. AND GILLES, R. (1984). Kinetic characteristics and specific activity of gill (Na^+-K^+) ATPase in the euryhaline crab *Eriocheir sinensis* during salinity acclimation. *Mar. Biol. Lett.* **5**, 35–45.

PERRY, H. M. (1984). A profile of the blue crab fishery of the Gulf of Mexico. In *Gulf States Marine Fisheries Commission*, Ocean Spring, Mississippi **9**, 1–80.

PIERCE, S. K. (1982). Invertebrate cell volume control mechanisms: A coordinated use of intracellular amino acids and inorganic ions as osmotic solute. *Biol. Bull. mar. biol. Bull., Woods Hole* **163**, 405–419.

PRESSLEY, T. A. AND GRAVES, J. S. (1983). Increased amino acid oxidation in the gills of blue crabs acclimated to dilute seawater. *J. exp. Zool*. **226**, 45–51.

RUMSEY, W. L., SCHLOSSER, C., NUUTINEN, E. M., ROBIOLIO, M. AND WILSON, D. F. (1990). Cellular energetics and the oxygen dependence of respiration in cardiac myocytes isolated from adult rat. *J. biol. Chem.* **265**, 15392–15399.

SABOURIN, T. D. (1983). The relationship between fluctuating salinity and oxygen delivery in adult blue crabs. *Comp. Biochem. Physiol.* **78**A, 109–118.

SAVAGE, J. P. AND ROBINSON, G. D. (1983). Inducement of increased gill Na^+–K^+-ATPase activity by a hemolymph factor in hyperosmoregulating *Callinectes sapidus*. *Comp. Biochem. Physiol*. **75**A, 65–69.

SIEBERS, D., LEWECK, K., MARKUS, H. AND WINKLER, A. (1982). Sodium regulation in the shore crab, *Carcinus maenas* as related to ambient salinity. *Mar. Biol*. **69**, 37–43.

TAGATZ, M. E. (1967). Noncommercial crabs of the genus *Callinectes* in St. Johns River, Florida. *Chesapeake Sci*. **8**, 202–203.

TOWLE, D. W. (1984). Membrane-bound ATPases in arthropod ion-transporting tissues. *Am. Zool*. **24**, 177–185.

TOWLE, D. W., PALMER, G. E. AND HARRIS, J. L. (1976). Role of gill Na^+–K^+-dependent ATPase in acclimation of blue crabs (*Callinectes sapidus*) to low salinity. *J. exp. Zool*. **196**, 315–322.

VANDEN AVYLE, M. J. AND FOWLER, D. L. (1984). Species profiles: Life histories and environmental requirements of coastal fishes and invertebrates (South Atlantic). *Fish and Wildlife Service* OBS-82/11.19. pp. 1–16.

WHITNEY, J. O. (1974). The effects of external salinity upon lipid synthesis in the blue crab *Callinectes sapidus* Rathbun and in the spider crab *Libinia emarginata* Leech. *Comp. Biochem. Physiol*. **49**A, 433–440.

WHITTAM, R. (1963). The interdependence of metabolism and active transport. In *The Cellular Functions of Membrane Transport* (ed. J. F. Hoffman), pp. 139–154. Englewood Cliffs, NJ: Prentice Hall.

WITTENBERG, B. A. AND WITTENBERG, J. B. (1985). Oxygen pressure gradients in isolated cardiac myocytes. *J. biol. Chem.* **260**, 6548–6554.

WITTENBERG, B. A. AND WITTENBERG, J. B. (1987). Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes. *Proc. natn. acad. Sci. U.S.A.* **84**, 7503–7505.