THE ANAEROBIC ENDPRODUCT LACTATE HAS A BEHAVIOURAL AND METABOLIC SIGNALLING FUNCTION IN THE SHORE CRAB *CARCINUS MAENAS*

BART DE WACHTER*, FRANZ-JOZEF SARTORIS AND HANS-OTTO PÖRTNER

Alfred Wegener Institut für Polar- und Meeresforschung, Biologie I, Ökophysiologie, Columbusstrasse, D-27568 Bremerhaven, Germany

Summary

This study presents the first evidence that, in invertebrates, the anaerobic endproduct lactate has an alarm signal function and induces metabolic and behavioural responses as in the anuran *Bufo marinus*. In support of this function, behavioural hypothermia was demonstrated in the shore crab *Carcinus maenas*. The animals moved to a cooler environment when exposed to hypoxic conditions. A decrease in preferred temperature of the same magnitude was also found when normoxic animals were injected with an iso-osmotic lactate solution resulting in a haemolymph concentration of approximately $12\,\mathrm{mmol\,l^{-1}}$. Under normoxic ($P_{O_2}=>18\,\mathrm{kPa}$) and moderately hypoxic conditions ($P_{O_2}=12\,\mathrm{kPa}$), injection of this lactate solution also caused a significant increase in the rate of oxygen consumption (100 and 50 % respectively). The increase in the rate of oxygen consumption was smaller and delayed, but lasted longer, under hypoxic conditions compared with normoxic conditions. Low but significant correlations between levels of lactate and levels of adrenaline, octopamine and tryptophan suggest the involvement of biogenic amines in the mediation of the signal.

Key words: Crustacea, emergency response, lactate, metabolic signal molecule, preferred temperature, oxygen consumption, biogenic amines, crab, *Carcinus maenas*, behavioural hypothermia.

Introduction

The shore crab *Carcinus maenas* is often exposed to dramatic changes in its physico-chemical environment. These include daily, seasonal and spatial changes in, among other variables, oxygen concentration ($2.8–67\,\mathrm{kPa}$) and temperature ($0–30\,°C$) (Morris and Taylor, 1983). Decapod crustaceans show many different physiological responses to environmental stressors (for reviews, see Mangum, 1983; McMahon and Wilkens, 1983; Vernberg, 1983). The physiological responses to hypoxia and temperature variation include changes in ventilation, circulation, haemocyanin oxygen-binding properties and in aerobic and anaerobic metabolism. However, information on circulating levels of biogenic amines in decapod crustaceans is scarce (Hoeger and Florey, 1989; Hoeger, 1990).

Lactate is the major anaerobic endproduct in crustaceans under hypoxic and anoxic conditions (Hill *et al.* 1991*a,b*). During a period of recovery from hypoxia, lactate levels are initially increased. Lactate is not excreted and only slowly remetabolised by gluconeogenesis (Hill *et al.* 1991*a*). Hill *et al.* (1991*a,b*) also showed that only part of the increased oxygen uptake associated with recovery from anoxia is concerned with the removal of lactate.

Besides physiological and metabolic adaptations, crustaceans also exhibit behavioural responses to hypoxia. The selection of a colder environment, i.e. behavioural hypothermia, has been described for crayfish *Procambarus simulans* in response to hypoxia (Dupré and Wood, 1988). Partial emersion under hypoxic conditions is also often observed in *Carcinus maenas* (Taylor *et al.* 1973, 1977; Taylor and Butler, 1973).

To elicit behavioural and physiological responses to adverse environmental situations, such as hypoxia, the animal must perceive this condition. The animal may sense this by a combination of several systems, i.e. peripheral or internal O_2 chemoreceptors, direct nervous response from the central nervous system or a response to circulating anaerobic endproducts. In the crayfish *Astacus leptodactylus*, the presence of peripheral O_2 receptors has been demonstrated (Massabuau and Burtin, 1984; Ishii *et al.* 1989). Wilkens *et al.* (1989) showed that parts of the nervous system of shore crabs, regulating ventilation and cardiac performance, are P_{O_2}-sensitive, indicating the presence of a direct nervous response. The importance of a system in which anaerobic endproducts elicit responses from animals in adverse situations is much less clear. As far as we know, no information is available on the signalling function of lactate in crustaceans. Pörtner *et al.* (1994) recently found that lactate that is formed at O_2 levels

below the critical P_{O_2} and released into the extracellular fluid functions in the toad *Bufo marinus* as a metabolic alarm signal in conjunction with catecholamines, eliciting both behavioural and metabolic emergency responses similar to those observed under hypoxia. This signalling mechanism would imply a central action of lactate *via* the mediation of catecholamines. The presence of this function of lactate in endothermic animals such as mammals and birds or in invertebrates remains to be established (Pörtner *et al.* 1994).

The goal of this study was to test the hypothesis that a similar signalling function for lactate exists in marine crustaceans and to answer the following questions. Do shore crabs show behavioural hypothermia? Can lactate infusion elicit this behaviour? Does lactate infusion induce a change in the rate of oxygen consumption? Is this response modified by external P_{O_2}? How do the time courses of the responses to lactate infusion (behavioural and metabolic) correlate with lactate catabolism under different levels of external P_{O_2}? Is there a correlation between changes in lactate levels and changes in the levels of biogenic amines?

Materials and methods

Animals

Shore crabs, *Carcinus maenas* (L.) (40–80 g; *N*=157), were obtained from commercial trawl-fishermen, who caught them in the German Bight of the North Sea in the autumn (September – November). After transport to the laboratory, they were held at densities of eight animals per 100 l in aquaria containing aerated recirculated North Sea water (35‰) at 13±0.5 °C and with a 12 h:12 h light:dark photoperiod. Crabs were kept in captivity for at least 2 weeks before experimental use. Nitrate, nitrite and ammonia levels in the sea water were checked at least weekly using sea water quality test kits (Tetra). Water was partially replaced when levels exceeded 6 mg l^{-1} nitrate, 0.1 mg l^{-1} nitrite and 0.25 mg l^{-1} ammonia. Animals were fed mussels *ad libitum* twice a week. The crabs were starved for at least 24 h before experimental use. Only intermoult animals were used in the experiments.

Measurement of preferred temperature

To measure the preferred temperature, a temperature gradient was constructed in a rectangular aquarium, 2 m long by 0.25 m wide by 0.5 m high, covered with a lid. Three coils, made of stainless steel, were placed inside this aquarium, each connected to a powerful waterbath (2000 W, Lauda) for temperature control. To maintain a low minimum temperature in the gradient, the entire experimental apparatus was placed in a cold room at 0 °C. The gradient was linearised, vertical stratification was minimised and, to control P_{O_2}, several aeration stones were placed on the bottom approximately 20 cm apart and the water was gently bubbled with either air or nitrogen delivered by a combination of mass flow controllers (MKS Multigas controller 647B GVS 19). The water depth was kept at 8 cm to maximise the stability of the gradient and to prevent the crabs from emerging from the water. The room

was kept dark to exclude photophobic behaviour, and potential crab refuge sites were avoided in the aquarium design. The temperature gradient along the length of the aquarium was from 6 to 25 °C. For analysis of the preferred temperature, animals were equipped with temperature probes (Omega Pt100 micro-thermocouples). The probes were swiftly connected to the crabs by attaching them under a piece of rubber band previously glued to the dorsal carapace of the crabs using cyanoacrylate glue. The probe was connected to a MacLab data-acquisition system (MacLab 4E) to register water temperature continuously. Simultaneously, P_{O_2} of the water was measured using a polarographic oxygen electrode (Eschweiler, Germany, no. 2000-100 4501). Data were sampled at 0.33 Hz and averaged over 1 min periods using the MacLab data-acquisition hardware and software (MacLab Chart V.3.3.3).

Two different experimental treatments were used. In the first treatment, animals were exposed to air-saturated sea water for 90 min, the gas mixture was then switched to nitrogen for 230 min and finally back to air for the remainder of the experiment (120 min). In the second treatment, animals that had been placed in the thermal gradient the night before were injected with a saline solution (460 mmol l^{-1} NaCl, 13 mmol l^{-1} KCl, 14 mmol l^{-1} CaCl$_2$, 20 mmol l^{-1} MgCl$_2$) after 90 min and a lactate solution 150 min later (460 mmol l^{-1} sodium lactate, 13 mmol l^{-1} KCl, 14 mmol l^{-1} CaCl$_2$, 20 mmol l^{-1} MgCl$_2$). The solution (500 µl) was injected into the base of the last walking leg. The composition of the solution was chosen to minimise the effects of fluctuating ion levels or an increase in proton level, which may influence catecholamine release, as has been reported for a hypoxic lactacidotic amphibian (*Ambystoma tigrinum*) (Talbot and Stiffler, 1991). To reduce the influence of potential circadian rhythms on the preferred temperature, all experiments were carried out at the same time of day.

Respiration measurements

The respiration measurements were performed using a flow-through respirometer (designed by F.-J.S. after Frier, 1976), placed in a thermostatted cold-room at 13 °C. This system (see Fig. 1) consisted of a maximum of six darkened respiration chambers connected to one polarographic oxygen electrode (Eschweiler, Germany, no. 2000-100 4501) *via* a rotating valve. During measurement of the outflow from a particular chamber, sea water was driven past the oxygen electrode by a peristaltic pump (Ismatec SW 5.01). Water flow through the five unconnected chambers was driven by gravity (at 80 ml min^{-1}). Incoming water, delivered from a constant-head tank, was bubbled with air or an air/N$_2$ mixture (P_{O_2}=12 kPa). Gas mixtures were delivered by a combination of mass flow controllers (MKS Multigas controller 647B GVS 19). Outflowing water was recirculated into the constant-head tank. Oxygen levels, either control values as found in the outflow of an empty chamber or experimental values from a chamber containing an animal, were measured with the oxygen electrode connected to an oxygen meter (Eschweiler M200). These data were sampled at 0.33 Hz using the MacLab data-
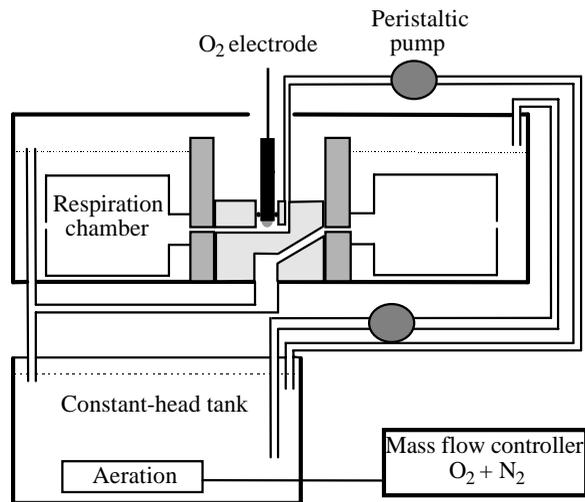
Fig. 1. Schematic drawing of a side view of the flow-through respirometer.

acquisition hardware (MacLab 4E) and software (MacLab Chart V.3.3.3). Steady state was reached within 2 min after switching the electrode to a new chamber. Seawater flow over the electrode was not influenced by the flow range used, and flow was chosen such that the P_{O_2} difference between inflowing and outflowing water was 10–15 % of that of the inflowing water.

To allow injection of saline or lactate solution, the animals (wet mass 64±12 g, mean ± S.E.M., N=13) were equipped with polyethylene catheters (i.d. 0.58 mm, o.d. 0.96 mm, length 30 cm) inserted in the coxa of the fifth pereiopod. Catheters were held in place with cyanoacrylate glue. To prevent loss of haemolymph, a latex membrane was glued to the coxa before the catheter was inserted through the epidermis. Surgery was conducted on submerged crabs and lasted for a maximum of 10 min. Animals were then placed into the darkened respiration chambers and allowed to recover and to adapt to the experimental chambers.

The experimental treatments used in the respiration experiments were as follows. In the first treatment, catheterised animals were acclimated in normoxic conditions (P_{O_2}=>18 kPa) to the respiration chamber for 45 h. At the end of the acclimation period, control rates of oxygen consumption in the animals were measured for at least 20 min. After this time, 500 μl of saline solution was injected, and the rate of oxygen consumption was measured for another hour. Approximately 24 h after saline injection, the rate of oxygen consumption was measured again before and after injection of the lactate solution.

In the second treatment, animals were tested under moderately hypoxic conditions. The P_{O_2} was reduced to 12 kPa after 20 h, and animals were acclimated to hypoxia for another 25 h. Control rates of oxygen consumption in the animals were measured for at least 20 min. Then, 500 μl of saline solution was injected, and the rate of oxygen consumption was measured for another hour. Five hours later, the rate of oxygen consumption

was again measured for 1 h. Approximately 24 h after saline injection, the rate of oxygen consumption was measured again before and after injection of the lactate solution. Lactate concentration in the haemolymph immediately after injection for both treatments was estimated to be 11.8±1.9 mmol l⁻¹ (mean ± S.D., N=13). This estimate, which assumes that the lactate remains in the haemolymph, is based on an estimated haemolymph volume of 30 % of the wet mass of the animal (Alspach, 1972; Gleeson and Zubkoff, 1977).

Lactate breakdown experiments

The metabolic removal of lactate from the haemolymph by catabolic processes (oxidation and gluconeogenesis) was studied by taking haemolymph samples over time from animals that had been injected with the lactate solution, under normoxic (>18 kPa) and moderately hypoxic (12 kPa) conditions. Individual crabs were isolated in darkened 2 l aquaria at 12±1 °C. Each aquarium was bubbled with either air or an air/N₂ mixture. After 15 h of acclimation to the aquarium, the first haemolymph sample was taken. One hour later, animals were injected with 500 μl of lactate solution at the base of the coxa of the first pereiopod. Haemolymph samples of approximately 0.5 ml were collected at the base of the last pereiopod at different times (0.25–36 h) after injection. Samples were collected in 1 ml plastic syringes using a hypodermic needle (0.6 mm×26 mm) inserted into the articular membrane at the base of the last walking leg. Samples that had not been collected within 20 s, including catching and handling, were discarded. Samples were immediately diluted in preweighed Eppendorf tubes containing 500 μl of ice-cold 0.28 mol l⁻¹ perchloric acid, and were weighed. Samples were then centrifuged for 4 min at 16 000 g and 2 °C (Eppendorf centrifuge 5402). The supernatant was frozen at −85 °C until analysis. Lactate concentrations were measured enzymatically (Bergmeyer, 1984).

Measurements of lactate accumulation

Crabs were individually placed in darkened 2 l aquaria at 12 °C. The aquaria were placed in a large vessel under temperature control (Julabo FP30). Each aquarium was bubbled with either air or an air/N₂ mixture. After 15 h of acclimation to the aquarium under either normoxia (>18 kPa) or hypoxia (8.6±0.4 kPa), haemolymph samples (0.5–1 ml) were collected and treated as described above. The supernatant was divided between two Eppendorf tubes, frozen in liquid N₂ and stored at −85 °C until analysis for lactate (see above) or biogenic amines (see below). Three different experiments were performed. In the first, animals were subjected to a temperature decrease from 12 to 0 °C under normoxia. The second series investigated animals exposed to hypoxia at 12 °C, and the third series included animals exposed to hypoxia and to a temperature decrease from 12 to 0 °C. The temperature decrease from 12 to 0 °C took approximately 4 h. Samples were collected prior to and 3, 7, 15 and 24 h after the start of the temperature decrease (i.e. after 15, 18, 22, 30 and 39 h in the experimental aquarium).

Analysis of biogenic amines

Biogenic amine concentrations were determined in the supernatant of the same samples taken from animals exposed to low temperature and/or hypoxia (see above). The supernatant was thawed at 4 °C, diluted by a factor of between 4 and 6 with $0.1 \, mol \, l^{-1}$ perchloric acid and filtered through a Millex-GV4 filter (Millipore, $0.22 \, \mu m$). Samples were thawed and kept in the dark until analysis. HPLC with electrochemical detection was used for determination of biogenic amine levels. The isocratic chromatographic system consisted of a Pharmacia LKB 2249 pump with a constant flow of $1 \, ml \, min^{-1}$, an injection valve (Rheodyne 9725i) with a $100 \, \mu l$ loop, a reversed-phase C18 column (CSC spherisorb ODS2, $5 \, \mu m$, $25 \, cm \times 0.46 \, cm$ column) with a guard column (BioRad microgram ODS 5S) and an electrochemical detector (Chromsystems ECD 41000) with a glassy carbon electrode set to a potential of $1000 \, mV$ and a Ag–AgCl reference electrode. The mobile phase consisted of $75 \, mmol \, l^{-1}$ NaH_2PO_4, $0.35 \, mmol \, l^{-1}$ 1-octane-sulphonic acid, $10 \, \mu mol \, l^{-1}$ EDTA, $9 \, \%$ (v/v) methanol and $4 \, \%$ (v/v) acetonitrile, pH 4.0, degassed and filtered through a $0.2 \, \mu m$ nylon filter. Chemicals and amine standards were obtained from Sigma Chemicals. Calibration curves for standards were prepared from stock solutions kept in the dark at 4 °C. The amines analysed were noradrenaline, adrenaline, octopamine, dopamine, serotonin, tryptophan and tyramine. The compounds were identified by retention times of standard mixtures and by standard addition experiments. Amine concentration was calculated from the peak area compared with calibration curves for standard solutions (single standards and mixtures of standards) and checked by standard addition experiments.

Data were analysed using the software packages Statistica and SuperAnova and parametric (repeated-measures analysis of variance, ANOVA; contrast analysis; Pearson product correlation; Bartlett's test for homogeneity of variances) and non-parametric (Spearman rank correlation; Mann–Whitney U-test; Kolmogorov two-sample test) techniques. Differences were considered significant at $P < 0.05$.

Results

Preferred temperature

Initially, shore crabs showed behavioural hyperthermia (Fig. 2). Animals placed in the temperature gradient consistently selected an initial preferred temperature of 19–20 °C, although their acclimation temperature was 12–13 °C. When the P_{O_2} was reduced, the animals moved to a significantly lower temperature (Pearson product correlation test; $r^2 = 0.59$; $P < 0.001$). Exposure to anoxia resulted in a decrease in the preferred temperature of approximately 3 °C after 2 h. Return to normoxic values resulted in a significant (Pearson product correlation test; $r^2 = 0.57$; $P < 0.001$) increase in the preferred temperature. A return to the pre-treatment preferred temperature, however, was not accomplished within 2 h.

Animals injected with saline under normoxic conditions showed no significant change in their preferred temperature (repeated-measures ANOVA; $P > 0.05$) (Fig. 3). When
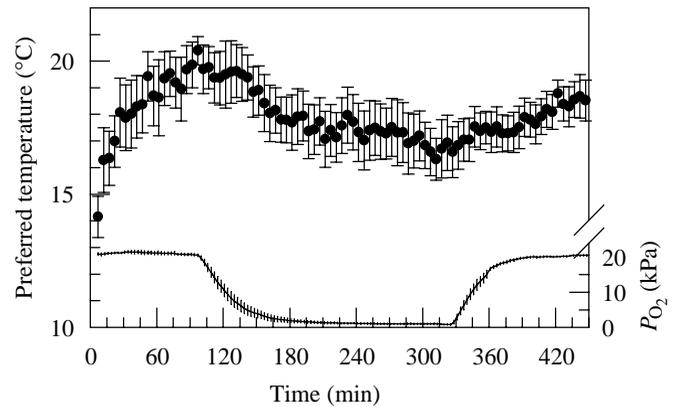
Fig. 2. Preferred temperature of *Carcinus maenas* and P_{O_2} in a temperature gradient (6–25 °C) over time. Data points represent mean ± S.E.M. ($N=8$). Significant correlations were found between the decrease in P_{O_2} and the decrease in preferred temperature and between the increase in P_{O_2} and the increase in preferred temperature ($P < 0.05$).

subsequently injected with lactate, they showed a significant (repeated-measures ANOVA with contrast analysis; $P < 0.01$) decrease in preferred temperature. Although the response was transient, since the preferred temperature started to increase slowly after 30 min, pre-treatment preferred temperature was not reached within 4.5 h.

Oxygen consumption

The rate of oxygen consumption doubled after *C. maenas* had been injected with saline or lactate (repeated-measures ANOVA with contrast analysis; $P < 0.05$) (Fig. 4). Comparison of the responses to lactate and saline showed that there was no significant difference in the control rate of oxygen consumption, i.e. before injection. This indicates that the rates
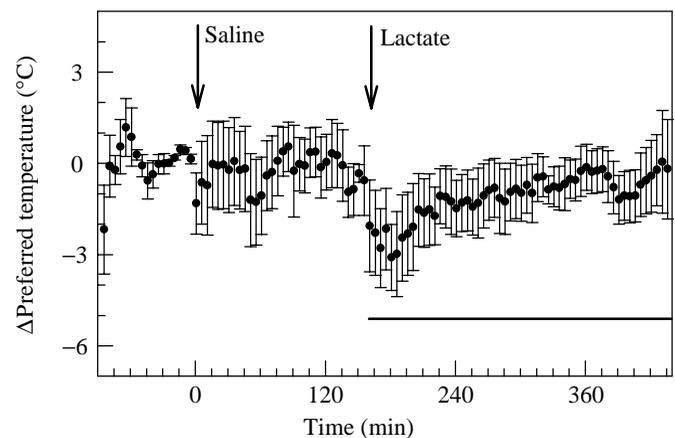
Fig. 3. The change in preferred temperature of *Carcinus maenas* in a temperature gradient gradient from 6 to 25 °C as a function of time. The times at which $500 \, \mu l$ of saline or lactate solution was injected are indicated by the arrows. Data points represent mean ± s.e.m. ($N=10$). Significant changes in preferred temperature compared with the value before injection ($t < 0 \, min$) are indicated by the horizontal line ($P < 0.05$).
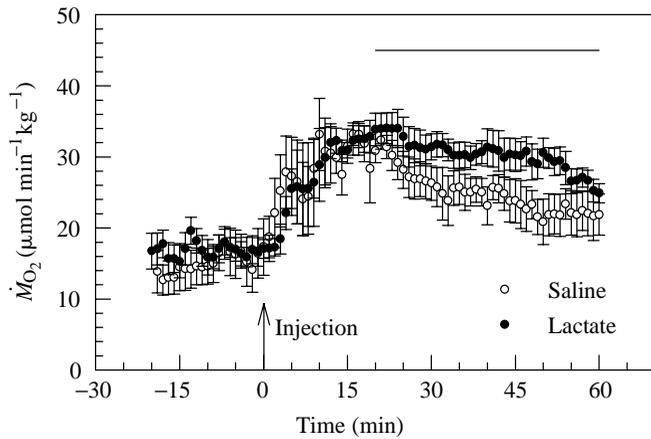
Fig. 4. Rate of oxygen consumption ($\dot{M}_{O_2}$) of *Carcinus maenas* as a function of time in a flow-through respirometer before and after injection of 500 µl of saline solution or lactate solution and under normoxic conditions (P_{O_2}=>18 kPa, 12 °C). Data points represent means ± S.E.M. (*N*=6). Significant differences in response between the two injections are indicated by the horizontal line (*P*<0.05).
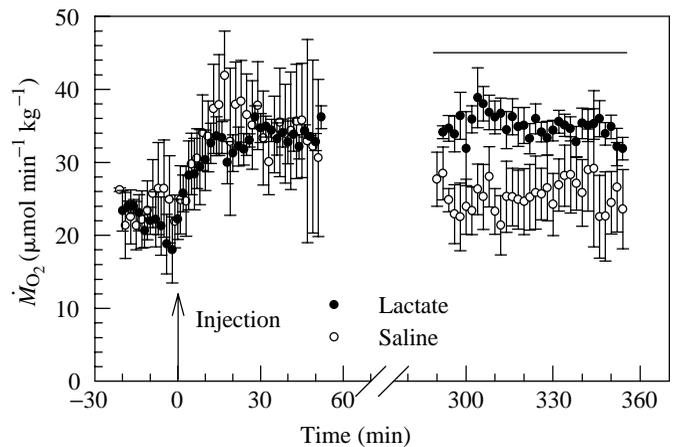

Fig. 5. Rate of oxygen consumption ($\dot{M}_{O_2}$) of *Carcinus maenas* as a function of time in a flow-through respirometer before and after injection of 500 µl of saline solution or lactate solution and under hypoxic conditions (P_{O_2}=12 kPa, 12 °C). Data points represent mean ± S.E.M. (*N*=5). Significant differences in response between the two injections are indicated by the horizontal line (*P*<0.05).

of oxygen consumption had returned to control levels within 24 h after saline injection, since lactate was injected 24 h after the saline injection. During the first 20 min after each injection, there was no difference in the rate of oxygen consumption between lactate- and saline-injected crabs. However, the rate of oxygen consumption 20 min after lactate injection was significantly (Kolmogorov two-sample test; *P*<0.05) elevated for the next 40 min compared with that of saline-injected animals. This means that oxygen consumption rates decreased faster after saline injection than after lactate injection.

During moderate hypoxia (P_{O_2}=12 kPa), a significant 1.5-fold increase in the rate of oxygen consumption occurred following injection of saline or lactate (repeated-measures ANOVA with contrast analysis; *P*<0.05) (Fig. 5). This increase was smaller than that under normoxic conditions (Kolmogorov two-sample test; *P*<0.05). No significant difference was found between the mean rate of oxygen consumption before saline injection and before lactate injection. This means that, as in normoxic animals, the rates of oxygen consumption had returned to control levels within 24 h after saline injection.

Respiration rates during the first 60 min following injection did not differ between lactate-injected and saline-injected specimens (Fig. 5), but after 5 h, the rates of oxygen consumption of lactate-injected specimens were significantly higher (Kolmogorov two-sample test; *P*<0.05).

After 5 h, the rate of oxygen consumption of animals injected with saline was not significantly different from the pre-injection value (repeated-measures ANOVA; *P*>0.05). Another important difference between the two treatments is the difference in variability. Animals injected with lactate showed a clearly reduced variability compared with saline-injected animals (Bartlett; *P*<0.05).

Lactate breakdown

Animals which had been injected with the same amount of

lactate as in previous experiments (preferred temperature and oxygen consumption), showed a slow catabolism of haemolymph lactate. Initial lactate concentrations were calculated on the basis of a haemolymph volume of 30 % of the wet mass. These values, which also imply a homogeneous distribution of lactate in the whole haemolymph volume, were not significantly different from the values measured after 0.25 or 0.5 h (Mann–Whitney *U*-test; *P*>0.05). After 1 h, lactate levels under normoxia were significantly higher than those under hypoxia, possible because of the higher initial concentration (Mann–Whitney *U*-test; *P*<0.05) (Fig. 6). However, after 4 h,
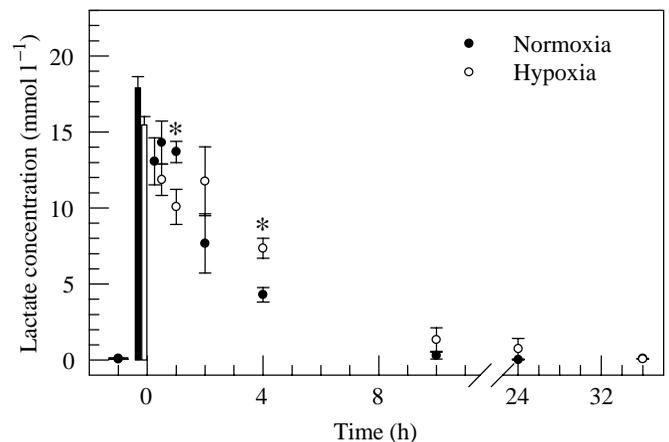

Fig. 6. Lactate concentrations in the haemolymph of *Carcinus maenas* before and after injection of 500 µl of lactate solution under normoxic (P_{O_2}=>18 kPa) (filled circles) and hypoxic (P_{O_2}=12 kPa) (open circles) conditions as a function of time (at 12 °C). Data points represent mean ± S.E.M. (*N*=3–6). The vertical bars represent calculated haemolymph concentrations immediately after injection (filled column, normoxic animals; open column, hypoxic animals). Significant differences between normoxia and hypoxia are indicated with an asterisk (*P*<0.05).
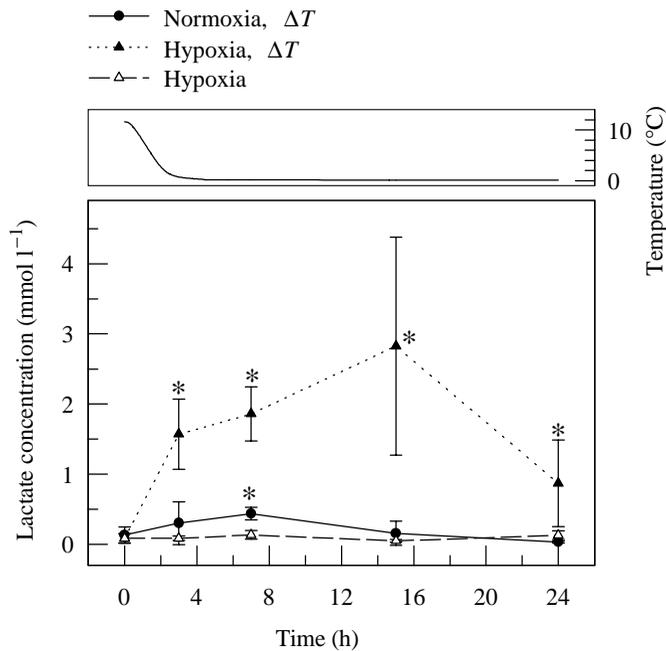
Fig. 7. Lactate concentrations in the haemolymph of *Carcinus maenas* as a function of time. Filled triangles represent means ± S.D. (*N*=6) for animals exposed simultaneously to a temperature decrease and hypoxia (8.6 kPa). Filled circles represent means ± S.D. (*N*=5–6) for animals exposed simultaneously to a temperature decrease and normoxia (>18 kPa). Open triangles represent means ± S.D. (*N*=3–6) for animals exposed to hypoxia (8.6 kPa) at a constant temperature (12 °C). Significant differences from control values (0 h) are indicated with an asterisk (*P*<0.05).
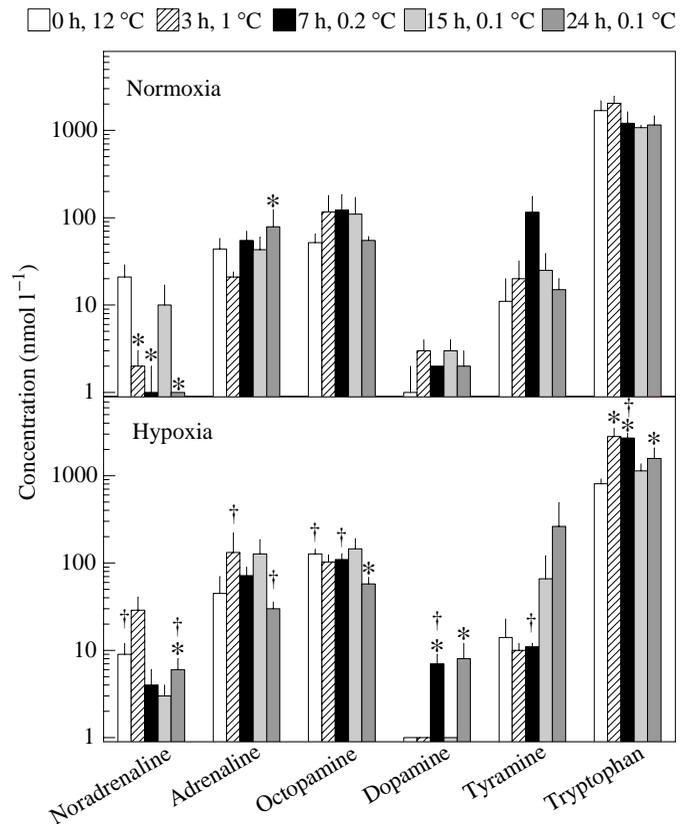

Fig. 8. Levels of noradrenaline, adrenaline, octopamine, dopamine, tyramine and tryptophan in the haemolymph of *Carcinus maenas* after 0, 3, 7, 15 and 24 h of exposure to a temperature decrease under normoxia (P_{O_2}=>18 kPa) (A) or hypoxia (P_{O_2}=8.6 kPa) (B). Bars represent means + S.E.M. (*N*=4–6). The detection limit was 3 nmol l^{-1}. An asterisk indicates a significant difference from the respective control value (0 h) (*P*<0.05). A dagger indicates a significant difference between the amine level under normoxia and the level under hypoxia (*P*<0.05).

lactate levels under normoxia were significantly lower than those under hypoxia (Mann–Whitney *U*-test; *P*<0.05). This indicates that the rate of lactate utilization is higher under normoxia than under hypoxia. After 10 h, lactate levels for both normoxia- and hypoxia-exposed animals were not significantly different from control values (Mann–Whitney *U*-test; *P*>0.05).

Lactate production

Fig. 7 shows lactate concentrations in the haemolymph of animals exposed to a temperature decrease under normoxia, a temperature decrease under hypoxia and during hypoxia without a temperature change. A temperature decrease under normoxic conditions resulted in a significant (Mann–Whitney *U*-test; *P*<0.05) increase (2.7-fold) in lactate concentration after 7 h (0.37±0.19 mmol l^{-1}) compared with control values (0.14±0.12 mmol l^{-1}). After 15 h, lactate concentrations had returned to control values. No significant change in lactate level was noted during hypoxic exposure at 12 °C. However, a temperature reduction in hypoxic (8.6 kPa) animals resulted in a significant (Mann–Whitney *U*-test; *P*<0.01) increase (up to 36-fold) compared with control values. Maximum lactate levels (2.83±1.55 mmol l^{-1}) were reached after 15 h, after which time they decreased again, but they were still elevated (0.87±0.62 mmol l^{-1}) after 24 h compared with control values (0.09±0.06 mmol l^{-1}).

Biogenic amines

Fig. 8 presents the levels of the different biogenic amines detected in the haemolymph of animals exposed to hypoxia (8.6 kPa) or normoxia and a simultaneous temperature reduction.

A small but significant positive correlation was found between the levels of adrenaline, octopamine and tryptophan and the level of lactate (Spearman rank-correlation; *r*=0.36, *P*<0.01 for adrenaline; *r*=0.31, *P*<0.05 for octopamine, and *r*=0.30, *P*<0.05 for tryptophan; Fig. 9).

In comparison with control values (0 h), significant reductions (Kolmogorov–Smirnov two-sample test; *P*<0.05) were found in the levels of noradrenaline (3 h, 7 h, 24 h), and a significant increase (*P*<0.05) in the level of adrenaline (24 h) under normoxia in combination with temperature decrease (Fig. 8). Hypoxic animals showed a significant reduction in the levels of noradrenaline (24 h) and octopamine (24 h), and a significant increase in the levels of dopamine (7 h, 24 h) and tryptophan (3 h, 7 h, 24 h), after a temperature decrease.
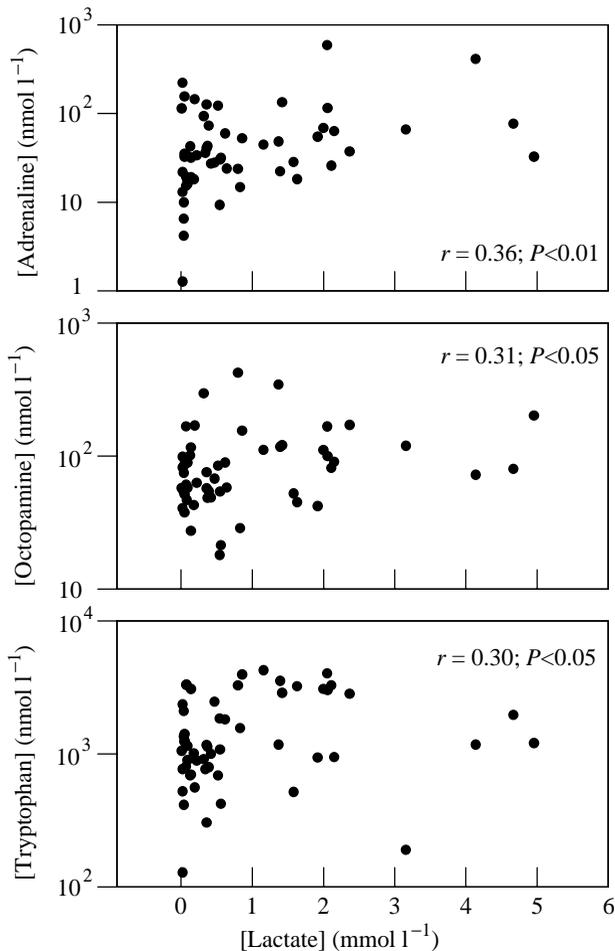
Fig. 9. Correlations between lactate levels and levels of adrenaline (A), octopamine (B) and tryptophan (C) in the haemolymph of *Carcinus maenas* for all animals.

Comparison of normoxic animals with hypoxic animals showed significant differences (Kolmogorov–Smirnov two-sample test; $P<0.05$) in the levels of noradrenaline (a decrease at 0 h, an increase at 24 h), adrenaline (an increase at 3 h, a decrease at 24 h), octopamine (an increase at 0 h, a decrease at 7 h), dopamine (an increase at 7 h), tyramine (a decrease at 7 h) and tryptophan (an increase at 7 h). Serotonin could be detected in only five of the 56 samples, and dopamine levels were also often below detection limits. The high level of inter-individual variability found in the biogenic amine levels in haemolymph samples should also be noted, together with the fact that noradrenaline (24 h) was not detected under normoxia, whereas dopamine was not detected during the hypoxic exposure experiment at 0 h, 3 h and 15 h.

Discussion

The initial temperature selection by the crabs brought into the temperature gradient under normoxia may be explained in the light of findings with different crayfish species. In *Procambarus spiculifer*, *P. clarkii* and *Cambarus latimanus*,

the preferred temperature in the laboratory matched the temperature in the natural environment encountered during the months of maximum physical activity (Taylor, 1984). *Procambarus* sp. also exhibited a circadian cycle in their preferred temperature matching the temperature cycles in their habitat. Acclimation temperature and starvation had no significant effect on the preferred temperature in several crayfish species (Crawshaw, 1983; Espina *et al.* 1993). The initial temperature selected by *C. maenas* also corresponds better with water temperature in the summer months than with the acclimation temperature (Morris and Taylor, 1983).

Behavioural hypothermia

Shore crabs showed behavioural hypothermia when exposed to hypoxia. While this response is well known in both homeothermic and ectothermic vertebrates, this is only the second time that it has been described for an invertebrate (Dupré and Wood, 1988). The decrease in preferred temperature in the shore crab was of similar magnitude to that in a hypoxic goldfish *Carassius auratus* (Crawshaw *et al.* 1989), but much smaller than the decrease seen in the crayfish *Procambarus simulans* (Dupré and Wood, 1988). Behavioural hypothermia has several advantages for an aquatic ectothermic animal: a reduction in metabolic rate, a decrease in critical oxygen concentration, which is characterised by the onset of anaerobic metabolism (Pörtner and Grieshaber, 1993; Branco *et al.* 1993) and an increase in oxygen-binding capacity of the oxygen carrier with decreasing temperature. All of these responses increase the hypoxia-tolerance of the animal. Injection of shore crabs with lactate, resulting in a haemolymph lactate concentration similar to that seen under severe hypoxia (Lallier *et al.* 1987; Taylor *et al.* 1977; Hill *et al.* 1991*b*), also caused a decrease in the preferred temperature. Initially, this decrease occurred to a similar extent (3 °C) to that during severe hypoxia and to the same extent as the decrease found in *Bufo marinus* after injection of iso-osmotic sodium lactate (Pörtner *et al.* 1994). As with *B. marinus*, this finding supports the hypothesis that lactate alone can induce behavioural hypothermia and may act as a metabolic alarm signal.

The time course of the behavioural response to lactate injection may be interpreted as being related to the time course of normoxic lactate breakdown if it is assumed that there is a threshold concentration of lactate in the haemolymph above which lactate becomes effective at a central level (see below). The effect of temperature should be taken into account. Although the shore crabs did show behavioural hypothermia after lactate injection, the selected temperature was still 3.5 °C higher than the temperature at which the lactate breakdown experiment was performed, causing the lactate effect to disappear earlier in the behavioural than in the lactate breakdown experiment.

Additionally, the almost immediate onset of an increase in preferred temperature in animals which recover from severe hypoxic exposure may indicate that other mechanisms are also involved in regulating preferred temperature. Catecholamines

may be a good candidate for contributing to the behavioural hypothermia response (see below). Alternatively, the oxygen-dependent catabolism of amines may be involved or O_2 sensors could affect the behaviour by sensing increased oxygen levels.

Oxygen consumption

During both normoxia and moderate hypoxia, lactate injection resulted in increased rates of oxygen consumption (Figs 4, 5). The difference between the responses to saline and lactate injections indicates that lactate alone caused the long-term increase in rates of oxygen consumption. The difference between normoxic and hypoxic animals in the magnitude of the response, immediately after injection, is probably related to the larger aerobic metabolic scope at higher O_2 levels. The P_{O_2} in these experiments was above the critical oxygen tension (Taylor *et al.* 1973, 1977) and does not result in anaerobic metabolism and lactate production. The rapid decline in the rate of oxygen consumption in normoxic animals may be related to the circulating level of lactate, although at first sight lactate removal is too slow and poorly correlated with $\dot{M}_{O_2}$ (Fig. 6). Also, the long-term response of hypoxic animals to lactate is probably due to the delayed decrease in lactate levels. The differences in time course can be explained if we assume that a minimum threshold level must be reached for lactate to become effective as a metabolic signal; this level may be higher than the $1.5\,\mathrm{mmol\,l^{-1}}$ estimated for *Bufo marinus* (Pörtner *et al.* 1994). In accordance with this conclusion, haemolymph lactate level fell below the threshold level faster in normoxic than in hypoxic specimens. However, the data set is not as clear as that for *Bufo marinus*, and we cannot exclude the possibility that the lactate response may be more complex in *C. maenas*, where the threshold is either set at a higher level than in *B. marinus* or obscured by concentration gradients building up within the circulatory system.

The increase in rates of oxygen consumption after lactate infusion also contrasts with the typical pattern of oxygen consumption during progressive hypoxia in *C. maenas*. In this species, a decrease in ambient P_{O_2} typically leads to a slow and then, below the critical P_{O_2}, sharp decrease in $\dot{M}_{O_2}$. *C. maenas* does not show the increase in rate of oxygen consumption at the critical P_{O_2} that is seen in *B. marinus* (Pörtner *et al.* 1991), although both ventilation and cardiac output increase (Taylor, 1976; Taylor *et al.* 1977; Booth and McMahon, 1985). The response to lactate is smaller in hypoxic (above the critical P_{O_2}) than in normoxic animals (Figs 4, 5), suggesting that, in contrast to the situation in *B. marinus*, the aerobic scope is too small in *C. maenas* to allow for an increase in rates of oxygen consumption when lactate production sets in as the critical P_{O_2} is reached.

It might be argued that the process of lactate removal is responsible for the increase in metabolic rate. In crustaceans, oxidative catabolism and gluconeogenesis are the major routes for lactate removal (Hill *et al.* 1991a,b). Gluconeogenesis would only imply an increase in energy turnover. Any use of lactate in oxidative catabolism would therefore follow the energy requirements of the organism and would not, by itself, explain an increase in metabolic rate (the use of other substrates would consequently be reduced) (see Pörtner *et al.* 1994). This is supported by the findings of Hill *et al.* (1991a,b) who demonstrated that, during recovery from anoxia, only part of the increased oxygen uptake is associated with lactate removal. The increased rate of oxygen consumption during recovery from anoxia is not only of the same magnitude as that in lactate-injected animals but is also transient. These results also appear to contrast with the much slower time course of lactate removal (Booth and McMahon, 1985; Lallier *et al.* 1987; Hill *et al.* 1991a,b) unless, again, a threshold level is postulated above which lactate exerts its stimulatory effect.

Lactate dynamics and biogenic amines

An acute temperature decrease resulted in a small and transient, but significant, increase in haemolymph lactate levels. In contrast, moderate hypoxia at a constant temperature did not result in any lactate production. The combination of the temperature decrease with hypoxia resulted in a drastic increase in lactate level. This indicates that there is a synergistic effect of temperature decrease and hypoxia, emphasising that the ventilatory capacity and blood oxygen transport in crustaceans, as in some other ectothermic animals, may limit their ability to tolerate low temperatures (Mauro and Mangum 1982a,b; Zielinski and Pörtner, 1996; Sommer and Pörtner, 1996). Although the temperature in our experiments was very low, it is not outside the natural temperature range of the shore crab. Morris and Taylor (1983) reported water temperatures as low as $0.5\,°C$ in rockpools at the Scottish coast, while the northern distribution limit of *Carcinus maenas* is the north of Norway and Iceland.

The positive correlation between lactate levels and the levels of adrenaline suggests that catecholamines may play a role in the emergency response. However, from these experiments, it is not possible to link directly changes in catecholamine levels with lactate-induced increases in rates of oxygen consumption and behavioural hypothermia. Nevertheless, these data are consistent with the results found for *B. marinus*, where adrenergic antagonists blocked the lactate-induced increase in $\dot{M}_{O_2}$. In goldfish, the presence of ethanol and the release of noradrenaline have been assumed to be linked, since both induce behavioural hypothermia (Wollmuth *et al.* 1987).

The significant correlations between both tryptophan and octopamine levels and lactate levels also suggest the involvement of monoamines in the lactate-induced emergency response. Changes in the levels of tryptophan, the precursor of serotonin, and octopamine can change the behaviour of crustaceans. Although serotonin was detected in only a few samples, it is known that serotonin induces tonic flexion of the tail muscle and aggressive behaviour in the lobster (Harris-Warrick *et al.* 1989). Octopamine induces muscle extension and behavioural submission. Monoamines are also known to modulate the sensitivity in the escape tailflip reflex in crayfish (Glanzman and Krasne, 1986). Both serotonin and octopamine are known to affect ventilation and circulation and may play a

role in the response to handling stress in the shore crab (Wilkens *et al.* 1985; Wilkens and McMahon, 1992).

The results presented above indicate that lactate does indeed have a signalling function in crustaceans. This anaerobic endproduct helps the animal to sense unfavourable conditions. As such, it is an emergency signal which results in behavioural and metabolic changes, probably mediated by changes in biogenic amine levels. The behavioural change is a decrease in the preferred temperature of the animal, optimising its energy balance and oxygen transport. The signalling function seems to be performed independently of the ambient oxygen tension, but the metabolic response may be limited by the aerobic scope. Future experiments should include measurements of the threshold level of lactate required to elicit the metabolic and behavioural emergency response. Experiments using amine blockers and measurements of changes in the levels of biogenic amines after lactate infusion should be performed to confirm the role of biogenic amines in this emergency response model.

The use of lactate as an anaerobic endproduct may be an evolutionary step forward compared with the use of other glycolytic endproducts such as opines, which are found in other invertebrate groups (Pörtner *et al.* 1994). The major advantage of lactate over opines is that lactate is distributed in a pH-dependent manner over different body compartments and accumulates predominantly in the body fluid, whereas opines are recycled intracellularly (Pörtner, 1993).

It still remains to be demonstrated that the signalling function of lactate exists in endothermic animals or in other lactate-forming invertebrate groups.

References

ALSPACH, G. S. (1972). Osmotic and ionic regulation in the Dungeness crab *Cancer magister*, Dana. PhD thesis, Oregon State University, Corvallis.

BERGMEYER, H. U. (1984). *Methods of Enzymatic Analysis*. Third edition. Vols 1–12. Weinheim: Verlag Chemie.

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.

BRANCO, L. G. S., PÖRTNER, H. O. AND WOOD, S. C. (1993). Interaction between temperature and hypoxia in the alligator. *Am. J. Physiol.* **265**, R1339–R1343.

CRAWSHAW, L. I. (1983). Effects of thermal acclimation and starvation on temperature selection and activity in the crayfish, *Orconectes immunis*. *Comp. Biochem. Physiol.* A **74**, 475–477.

CRAWSHAW, L. I., WOLLMUTH, L. P. AND O'CONNER, C. S. (1989). Intracranial ethanol and ambient anoxia elicit selection of cooler water by goldfish. *Am. J. Physiol.* **256**, R133–R137.

DUPRÉ, R. K. AND WOOD, S. C. (1988). Behavioral temperature regulation by aquatic ectotherms during hypoxia. *Can. J. Zool.* **66**, 2649–2652.

ESPINA, S., DIAZ HERRERA, F. AND BÜCKLÉ RAMIREZ, L. F. (1993). Preferred and avoided temperatures in the crawfish *Procambarus clarkii* (Decapoda, Cambaridae). *J. therm. Biol.* **18**, 35–39.

FRIER, J. O. (1976). Oxygen consumption and osmoregulation in the isopods *Sphaeroma hookeri* Leach and *S. rugicauda* Leach. *Ophelia* **15**, 193–203.

GLANZMAN, D. L. AND KRASNE, F. B. (1986). 5,7-Dihydroxytryptamine lesions of crayfish serotonin-containing neurons: Effect on the lateral giant escape reaction. *J. Neurosci.* **6**, 1560–1569.

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.* **6**, 1560–1569.

HARRIS-WARRICK, R. M., FLAMM, R. E., JOHNSON, B. R. AND KATZ, P. S. (1989). Modulation of neural circuits in Crustacea. *Am. Zool.* **29**, 1305–1320.

HILL, A. D., STRANG, R. H. C. AND TAYLOR, A. C. (1991*a*). Radioisotope studies of the energy metabolism of the shore crab *Carcinus maenas* (L.) during environmental anoxia and recovery. *J. exp. mar. Biol. Ecol.* **150**, 51–62.

HILL, A. D., TAYLOR, A. C. AND STRANG, R. H. C. (1991*b*). Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery. *J. exp. mar. Biol. Ecol.* **150**, 31–50.

HOEGER, U. (1990). Fate of circulating serotonin in the hemolymph of the crayfish, *Orconectes limosus*. *Z. Naturforsh.* **45**c, 1053–1059.

HOEGER, U. AND FLOREY, E. (1989). Catecholamine degradation in the hemolymph of the chinese crab, *Eriocheir sinensis*. *Comp. Biochem. Physiol.* **92**C, 323–327.

ISHII, K., MASSABUAU, J.-C. AND DEJOURS, P. (1989). Oxygen-sensitive chemoreceptors in the branchio-cardiac veins of the crayfish, *Astacus leptodactylus*. *Respir. Physiol.* **78**, 73–81.

LALLIER, F., BOITEL, F. AND TRUCHOT, J. P. (1987). The effect of ambient oxygen and temperature on haemolymph L-lactate and urate concentrations in the shore crab *Carcinus maenas*. *Comp. Biochem. Physiol.* **86**A, 255–260.

MANGUM, C. P. (1983). Oxygen transport in the blood. In *The Biology of Crustacea*, vol. 5, *Internal Anatomy and Physiological Regulation*, chapter 7 (ed. L. H. Mantel), pp. 373–429. New York: Academic Press.

MASSABUAU, J.-C. AND BURTIN, B. (1984). Regulation of oxygen consumption in the crayfish *Astacus leptodactylus* at different levels of oxygenation: role of peripheral O2-chemoreception. *J. comp. Physiol.* B **155**, 43–49.

MAURO, N. A. AND MANGUM, C. P. (1982*a*). The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. *J. exp. Zool.* **219**, 179–188.

MAURO, N. A. AND MANGUM, C. P. (1982*b*). The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. II. Interspecific adaptations to latitudinal changes. *J. exp. Zool.* **219**, 189–195.

MCMAHON, B. R. AND WILKENS, J. L. (1983). Ventilation, perfusion and oxygen uptake. In *The Biology of Crustacea*, vol. 5, *Internal Anatomy and Physiological Regulation*, chapter 6 (ed. L. H. Mantel), pp. 289–372. New York: Academic Press.

MORRIS, S. AND TAYLOR, A. C. (1983). Diurnal and seasonal variation in physico-chemical conditions within intertidal rock pools. *Est. coast. Shelf Sci.* **17**, 339–355.

PÖRTNER, H. O. (1993). Multicompartmental analysis of acid–base and metabolic homeostasis during anaerobiosis: invertebrate and lower vertebrate examples. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (ed. P. W. Hochachka, P. L. Lutz, M. Rosenthal, T. J. Sick and G. van den Thillart), pp. 139–156. Boca Raton, FL: CRC Press.

PÖRTNER, H. O., BRANCO, L. G. S., MALVIN, G. M. AND WOOD, S. C.

(1994). A new function for lactate in the toad *Bufo marinus*. *J. appl. Physiol.* **76**, 2405–2410.

PÖRTNER, H. O. AND GRIESHABER, M. K. (1993). Critical P_{O_2}(s) in oxyconforming and oxyregulating animals: gas exchange, metabolic rate and the mode of energy production. In *The Vertebrate Gas Transport Cascade: Adaptations to Environment and Mode of Life* (ed. E. Bicudo), pp. 330–357. Boca Raton, FL: CRC Press.

PÖRTNER, H. O., MACLATCHY, L. M. AND TOEWS, D. P. (1991). Metabolic responses of the toad *Bufo marinus* to environmental hypoxia: an analysis of critical P_{O_2}. *Physiol. Zool.* **64**, 836–849.

SOMMER, A., KLEIN, B. AND PÖRTNER, H. O. (1996). Temperature induced anaerobiosis in two populations of the polychaete worm *Arenicola marina* (L.). *J. comp. Physiol.* (in press).

TALBOT, C. R. AND STIFFLER, D. F. (1991). Effects of hypoxia on acid–base balance, blood gases, catecholamines and cutaneous ion exchange in the larval tiger salamander (*Ambystoma tigrinum*). *J. exp. Biol.* **257**, 299–305.

TAYLOR, A. C. (1976). The respiratory responses of *Carcinus maenas* to declining oxygen tension. *J. exp. Biol.* **65**, 309–322.

TAYLOR, E. W. AND BUTLER, P. J. (1973). The behaviour and physiological responses of the shore crab *Carcinus maenas* during changes in environmental oxygen tension. *Neth. J. Sea Res.* **7**, 496–505.

TAYLOR, E. W., BUTLER, P. J. AND AL-WASSIA, A. (1977). Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. *J. comp. Physiol.* **122**, 391–402.

TAYLOR, E. W., BUTLER, P. J. AND SHERLOCK, P. J. (1973). The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. *J. comp. Physiol.* **86**, 95–115.

TAYLOR, R. C. (1984). Thermal preferred and temporal distribution in three crayfish species. *Comp. Biochem. Physiol.* **77**A, 513–517.

VERNBERG, F. J. (1983). Respiratory adaptations. In *The Biology of Crustacea*, vol. 8, *Environmental Adaptations*, chapter 1 (ed. F. J. Vernberg and W. B. Vernberg), pp. 1–33. New York: Academic Press.

WILKENS, J. L. AND MCMAHON, B. R. (1992). Intrinsic properties and extrinsic neurohormonal control of crab cardiac hemodynamics. *Experientia* **48**, 827–934.

WILKENS, J. L., MERCIER, A. J. AND EVANS, J. (1985). Cardiac and ventilatory responses to stress and to neurohormonal modulators by the shore crab *Carcinus maenas*. *Comp. Biochem. Physiol.* **82**C, 337–343.

WILKENS, J. L., YOUNG, R. E. AND DICAPRIO, R. A. (1989). Responses of the isolated crab ventilatory central pattern generators to variations in oxygen tension. *J. comp. Physiol.* B **159**, 29–36.

WOLLMUTH, L. P., CRAWSHAW, L. I. AND PANAYIOTIDES-DJAFERIS, H. (1987). Thermoregulatory effects of intracranial norepinephrine injections in goldfish. *Am. J. Physiol.* **253**, R821–R826.

ZIELINSKI, S. AND PÖRTNER, H. O. (1996). Energy metabolism and ATP free-energy change of the intertidal worm *Sipunculus nudus* below a critical temperature. *J. comp. Physiol.* (in press).