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EXTRACELLULAR AND INTRACELLULAR ACID–BASE STATUS IN THE FRESHWATER CRAYFISH *AUSTROPOTAMOBIUS PALLIPES* BETWEEN 1 AND 12 °C

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Summary

Freshwater crayfish, *Austropotamobius pallipes*, caught in the winter when water temperature was 5 °C, were acclimated in the laboratory to 5 °C for 1 week and to 1 and 12 °C for 1 month before haemolymph and tissue samples were taken for acid–base analysis. *In vivo*, haemolymph (extracellular) pH increased by 0.14 units between 5 and 1 °C but remained unchanged between 5 and 12 °C, giving an overall ΔpHe/Δt value of -0.009 pH units °C^{-1} and an [H$^+$] value of +3.3 nmol l^{-1}. Haemolymph withdrawn from crayfish acclimated to 1 °C and warmed anaerobically *in vitro* to 12 °C had a ΔpHe/Δt value of -0.010 pH units °C^{-1}. This was depressed compared with the values of -0.015 and -0.016 pH units °C^{-1} obtained from haemolymph withdrawn from crayfish held at 5 and 12 °C, respectively, but changes in H$^+$ concentration were similar in all three groups. There was little change in intracellular pH with rise in temperature in either claw muscle (ΔpHi/Δt= -0.003 pH units °C^{-1}) or hepatopancreas (ΔpHi/Δt= -0.006 pH units °C^{-1}). In the heart, ΔpHi/Δt paralleled that in the haemolymph (-0.010 pH units °C^{-1}), but the largest change was found in the abdominal muscle (-0.025 pH units °C^{-1}). In all tissues apart from claw muscle, there was a metabolic acidosis, with an increase in metabolic acid load as temperature rose from 1 to 12 °C. The depressed *in vivo* ΔpHe/Δt value and the differences in ΔpHi/Δt between the tissues were attributed to active regulation of pHi, possibly to control enzyme function in the intracellular compartments. Tissues that are functionally important in the winter months, such as abdominal muscle, followed alphastat regulation, whereas tissues that are less active in the winter, such as claw muscle and hepatopancreas, maintained pHi irrespective of temperature, resulting in a relatively acidotic pHi, which may serve to depress the activity of metabolic enzymes.

Key words: crayfish, *Austropotamobius pallipes*, acid–base balance, temperature.

Introduction

Environmental temperature is known to influence acid–base status in poikilothermic animals (e.g. Jackson, 1982; Cossins and Bowler, 1987). In the extracellular compartment, pH is alkaline and typically varies inversely with body temperature. The resulting temperature coefficients (ΔpHe/Δt) have been described as paralleling the temperature-related changes in the pH of pure water and of the dissociation constant for imidazole, the titratable group of histidine (Reeves, 1972; Rahn and Howell, 1978). Consequently, poikilotherms have been characterised as maintaining a constant relative alkalinity in their extracellular fluids (Rahn, 1967; Howell *et al.* 1970); the supposed advantage being an unchanging degree of ionisation of proteins and the α-imidazole group in particular, resulting in the maintenance of the integrity of protein function over a range of temperatures (the alphastat model of Reeves, 1972).

In aquatic crustaceans, the pHe of the extracellular compartment (i.e. the haemolymph) typically varies with temperature, with ΔpHe/Δt values ranging from -0.015 to -0.023 pH units °C^{-1} between 10 and 30 °C (Howell *et al.* 1973; Truchot, 1973, 1978, 1987; Cameron and Batterton, 1978; McMahon *et al.* 1978; Taylor, 1981; Cameron, 1986). There are fewer studies on the relationship between intracellular pH and temperature in aquatic crustaceans, but data available from animals in steady-state conditions show that pHi in several soft tissues, namely nerve cord, heart, abdominal and claw muscle in the crayfish (Rodeau, 1984; Gaillard and Malan, 1985) and the blue crab (Wood and Cameron, 1985), varies with temperature, having ΔpHi/Δt slopes of around -0.015 pH units °C^{-1}. Despite these similarities between ΔpHe/Δt and ΔpHi/Δt, a number of experiments on water-breathing crustaceans under a variety of different conditions have shown that pHi can vary between tissues independently of pHe (Gaillard and Malan, 1983; Wood and Cameron, 1985; Tyler-Jones and Taylor, 1988; Milligan *et al.* 1989; Wheatly and Henry, 1992), an observation which has also been reported in several fish species (Heisler *et al.* 1980; Heisler, 1986). Intracellular pH has also been shown not to vary with temperature in temperate aquatic poikilotherms

acclimatised to their seasonal temperatures. For example, in the American eel, there is little or no change in pHe and pHi of either white or red muscle between 5 and 15–20 °C as a result of relatively acidotic pHe and pHi values at 5 °C (Walsh and Moon, 1982). The accumulation of protons at low temperature was thought to reduce standard metabolic rate during the winter, when the animals are generally sluggish and inactive, by deactivation of intracellular enzymes, as described in hibernating mammals (Malan, 1985).

Seasonal effects on $\Delta pHe/\Delta t$ have also been observed in the freshwater crayfish *Austropotamobius pallipes*. *In situ* measurements revealed that there was no change in haemolymph pH in a wild population of crayfish as water temperatures fell in the winter months from 11 to 1 °C, when the animals were observed to be relatively inactive (Whiteley and Taylor, 1993). The maintenance of a constant pHe of approximately 7.9 at temperatures below 11 °C was explained in terms of the changing relationship between pH, P_{CO_2} and $[HCO_3^-]$ in balancing temperature-related changes in physico-chemical constants, suggesting active regulation of pHe. The consequences of this unchanging pHe for pHi regulation in the intracellular compartments of the metabolising tissues of winter crayfish have not been investigated. This clearly merits attention, since pHi may determine the specific activity of the enzyme-catalysed reactions of metabolism and, in turn, influence temperature-related changes in metabolic rate. Consequently, the aim of the present study was to investigate seasonal pH regulation in freshwater crayfish by acclimating winter animals to an increased or decreased temperature for 1 month in the laboratory. The resulting acid–base response in the haemolymph of these crayfish was followed *in vivo* and *in vitro* and was related to changes in intracellular pH in several tissues to determine the overall acid–base status of *A. pallipes* at low temperatures.

Materials and methods

Crayfish were obtained from the same wild population of *Austropotamobius pallipes* (L.) inhabiting Stowe Pool, Lichfield, South Staffordshire (Ordnance Survey Grid Reference SK101 122), as used in the previous *in situ* study (Whiteley and Taylor, 1993). Thirty-two adult males and two females, ranging in mass from 12 to 38 g and in carapace length from 39 to 55 mm, were collected by SCUBA divers in January 1993, when the water temperature was 5 °C, and transferred to holding tanks at Birmingham University.

On arrival, the animals were separated into three groups. Each group was held in fully aerated, dechlorinated Birmingham tapwater at 1, 5 or 12 °C. Crayfish held at 5 °C (mean mass 24.9±2.7 g, N=8) were given 1 week to recover from collection but no longer, as this was the acclimatisation temperature in the wild. At 1 °C (mean mass 19.1±2.1 g, N=5) and 12 °C (mean mass 25.3±3.2 g, N=8) crayfish were allowed 4 weeks to acclimate to the change in temperature. In all cases, the animals were maintained under conditions of 8 h:16 h light:dark. The animals held at 1 and 12 °C were fed once a week and were sampled 1 week after the last feed. Animals kept at 5 °C for 1 week were not fed.

Water characteristics

Titratable alkalinity of the holding water was measured on filtered samples (Millipore, 0.45 μm) using the method outlined by Rodier (1975). Ion levels in the water were determined by Permutit Company Limited (Permutit House, Middlesex, UK). The characteristics of dechlorinated Birmingham tapwater were as follows: $[Ca^{2+}]$= 0.50 mequiv l^{-1}, $[Mg^{2+}]$=0.50 mequiv l^{-1}, $[Na^+]$=0.57 mequiv l^{-1}, $[K^+] \leqslant 0.03$ mequiv l^{-1}, $[Cl^-]$=0.45 mequiv l^{-1}; titratable alkalinity=0.2–0.3 mequiv l^{-1}.

Experimental procedure

Postbranchial haemolymph samples of 0.3–0.7 ml were withdrawn anaerobically from the pericardial sinus of each crayfish, as described by Whiteley and Taylor (1993), for the immediate determination of extracellular acid–base status. The remainder of each haemolymph sample was then stored at −20 °C for the determination of L-lactate levels and for the *in vitro* investigation of closed-system acid–base changes with temperature. Haemolymph was sampled quickly (within 1 min) from submerged animals, with the minimum of disturbance. Each animal was then immediately killed and the following tissues were dissected out for the determination of intracellular pH (pHi): heart, abdominal muscle (deep flexor), claw muscle (closer) and hepatopancreas. The tissues were isolated rapidly and immediately freeze-clamped in aluminium tongs which had been pre-cooled in liquid nitrogen. The clamped samples were stored under liquid nitrogen prior to analysis.

An additional set of tissue samples was collected from a separate group of animals (mean mass 7.0±1.2 g, N=5), including individuals acclimated at 5 and 12 °C, for the determination of tissue water content. Samples of heart, hepatopancreas, claw muscle and abdominal muscle were blotted, weighed and dried in an oven at 80 °C until a constant mass was obtained.

Extracellular acid–base status

In vivo *measurements*

Postbranchial haemolymph samples were used for the immediate measurement of extracellular pH (pHe) and total CO_2. Haemolymph pH was determined using a glass capillary electrode (Radiometer E5021a) maintained at the appropriate acclimation temperature and connected to a Radiometer acid–base analyser (PHM71). For measurements at 1 °C, the pH electrode was calibrated using a phosphate buffer (pH 7.11 at 1 °C) and a borate buffer (pH 9.48 at 1 °C) to cover the range of measured values. For measurements at 5 and 12 °C, the electrode was calibrated using precision buffers (Radiometer S1500 and S1510, pH 6.951 and 7.497, respectively, at 5 °C). Consecutive readings of each haemolymph sample were taken until consistent values were obtained. Total CO_2 (C_{CO_2}) was determined on 50 μl subsamples using the technique described by Cameron (1971) in which a CO_2 electrode was inserted into

a 2 ml chamber filled with 0.01 mol l^{-1} HCl and maintained at 38 °C. The electrode was connected to a Radiometer PHM73 pH/blood gas monitor and calibrated with standard $NaHCO_3$ solutions of 5, 10 and 20 mmol l^{-1}.

Measured values of pHe and total CO_2 were used to calculate P_{CO_2} and $[HCO_3^-]$ levels in the haemolymph *via* the Henderson–Hasselbalch equation using (i) solubility coefficients for CO_2 (αCO_2) taken from values obtained for sea water by Murray and Riley (1971) and adjusted for temperature at a haemolymph $[Na^+]$ of 200 mequiv l^{-1} (value for winter crayfish, N. M. Whiteley, unpublished observations) and (ii) operational pK_1' values measured from winter crayfish and adjusted for temperature and pH using the regression equations given in a previous study (Whiteley and Taylor, 1993). In each case, individual pH values were converted into H^+ concentrations.

Haemolymph relative alkalinity ratios ($[OH^-]/[H^+]$) were calculated according to the equation:

$$[OH^-]/[H^+] = \text{antilog}[2(pH - pN)],$$

where pN is the pH of neutrality and is equal to 0.5pKw, the negative logarithm of the ionisation constant of water, as described by Howell *et al.* (1973). pN was taken to be 7.450 at 1 °C, 7.370 at 5 °C and 7.234 at 12 °C (Weast, 1993).

Haemolymph L-lactate concentrations were determined following a modification of the NAD/NADH technique (Sigma kit no. 826-UV) as described by Graham *et al.* (1983).

In vitro *experiments*

Changes in closed-system haemolymph acid–base status with temperature were examined by warming haemolymph samples anaerobically in glass syringes and withdrawing subsamples for the measurement of pH and total CO_2. Haemolymph samples from each group of crayfish held in dechlorinated Birmingham tapwater were used individually or pooled to a total volume of 2 ml and transferred to air-tight glass syringes containing glass beads. Samples from five animals at 1 °C, six animals at 5 °C and six animals at 12 °C were warmed from 1 °C to 5 °C and then to 12 °C, allowing a 20 min equilibration time at each stage, aided by gentle mixing of the samples. Haemolymph pH and total CO_2 were measured at 1 °C, 5 °C and 12 °C using the techniques described for the *in vivo* haemolymph measurements, with calibration performed at the appropriate temperature. Corresponding P_{CO_2} and HCO_3^- levels were calculated as described above.

Intracellular acid–base status

Intracellular pH (pHi) was determined on homogenised frozen tissue using the method described by Pörtner *et al.* (1990). In summary, the frozen tissues were each ground under liquid nitrogen to a fine powder using a pre-cooled pestle and mortar. Subsamples of powder (10–150 mg) were then rapidly transferred to a pre-weighed 0.5 ml Eppendorf tube containing 300 μl of inhibitor medium to stop further metabolism. This medium contained 130 mmol l^{-1} of potassium fluoride and 6 mmol l^{-1} of nitrilotriacetic acid, adjusted to pH 7 using potassium hydroxide, and was made up immediately before use. After reweighing, the Eppendorf tube containing the tissue was completely filled with a known volume of the inhibitor medium, stirred briefly with a mounted needle to release bubbles and capped tightly. The contents were well mixed on a Whirlimixer (Fisons) and centrifuged for about 15 s before consecutive pH readings of the supernatant were taken at the appropriate experimental temperature. Great care was taken to recap the Eppendorf tubes after each reading and to place the tubes on ice to minimise potential pH changes resulting from CO_2 loss.

Table 1. *Mean values for the tissue water content of the heart, hepatopancreas, abdominal muscle and claw muscle, along with corresponding values for extracellular fluid volume, obtained in other studies by measurement of inulin space*

Tissue	Tissue water content (g water 100 g^{-1} tissue)	ECFV (g water 100 g^{-1} tissue)	Reference for ECFV data
Heart	92.8±1.3	21.6±3.8 (6 °C) 22.1±6.2 (13 °C)	Gaillard and Malan (1985)
Hepato-pancreas	79.0±1.4	15.3±1.6 (15 °C)	S. C. R. de Souza (unpublished observations)
Abdominal muscle	82.0±0.3	15.7±0.2 (6 °C)	Gaillard and Malan (1985)
Claw muscle	79.3±0.8	10.5±0.1 (6 °C) 11.3±0.2 (13 °C)	Gaillard and Malan (1985)

Values are means ± S.E.M., N=5.
ECFV, extracellular fluid volume.

Measured values of tissue pH were corrected for the influence of the inhibitor medium (buffering and dilution effects) according to the equations of Pörtner *et al.* (1990), using the values for tissue water content and extracellular fluid volume (ECFV) displayed in Table 1. However, these corrections made no significant difference to the pHi values. Intracellular HCO_3^- values were calculated using the Henderson–Hasselbalch equation, assuming that the values for intracellular P_{CO_2}, αCO_2 and pK_1' were the same as those determined in the extracellular compartment. Similar assumptions were made by Gaillard and Malan (1983) and Wheatly *et al.* (1991). In addition, pHi values were converted into H^+ concentrations and the differences in $[H^+]$ between 1 and 12 °C were calculated for each tissue. The fraction of hydrogen ions added to the haemolymph buffers by metabolic acids (i.e. the metabolic acid load) was determined following the equation originally used by Wood *et al.* (1977):

$$\Delta H_m^+ = [HCO_3^-]_1 - [HCO_3^-]_{12} - \beta(pH_1 - pH_{12}),$$

where 1 and 12 refer to the two acclimation temperatures. Intracellular values were obtained from the values determined for *Pacifastacus leniusculus* by Wheatly *et al.* (1991).

Statistical analyses

Measured values are given as means ± S.E.M. with the number of observations in parentheses. Statistical differences between the means of the dependent variables were determined by one-factor analysis of variance (ANOVA). Multiple comparisons were carried out using the ANOVA Fisher's PLSD test. Differences were accepted as significant at the 95 % level of confidence (P<0.05). The relationships between temperature and acid–base variables in the extra- and intracellular compartments were subjected to linear regression analysis, as described in the text.

Results

Extracellular acid–base status

In vivo *measurements*

The relationship between the main haemolymph acid–base variables and acclimation temperature are shown in Fig. 1. At 5 ˚C, mean haemolymph pH (pHe) was 7.88±0.02 (N=7) which represented a mean $[H^+]$ of 12.83±0.70 nmol l^{-1} (N=7). Following a decrease in water temperature from 5 ˚C to 1 ˚C, pHe increased significantly to 8.02±0.03 (N=5) ($[H^+]$=9.60±1.49 nmol l^{-1}), but between 5 ˚C and 12 ˚C pHe remained unchanged with a value of 7.90±0.02 (N=8) ($[H^+]$=12.88±0.64 nmol l^{-1}) at 12 ˚C. Incorporation of all pHe values into a linear regression equation produced a temperature coefficient of −0.009 pH units ˚C^{-1} between 1 ˚C and 12 ˚C, which marked an increase in $[H^+]$ by 3.3 nmol l^{-1} over the 11 ˚C change in temperature (Table 2). The relative alkalinity ratio ($[OH^-]/[H^+]$) in the haemolymph ranged from 14.5±1.9 (N=5) at 1 ˚C to 11.1±1.3 (N=7) at 5 ˚C and back to 15.7±0.9 (N=8) at 12 ˚C.

Between 1 and 12 ˚C, there was a decline in total CO_2 levels in the haemolymph from 7.16±0.92 mmol l^{-1} (N=5) at 1 ˚C to 5.35±0.68 mmol l^{-1} (N=8) at 12 ˚C, while calculated levels of $[HCO_3^-]$ fell from 7.05±0.9 (N=5) to 5.24±0.66 mequiv l^{-1} (N=8) over the same temperature range (Fig. 1), but none of these changes was significant. Overall, haemolymph P_{CO_2} levels increased between 1 and 12 ˚C by 0.05 kPa (Table 2),

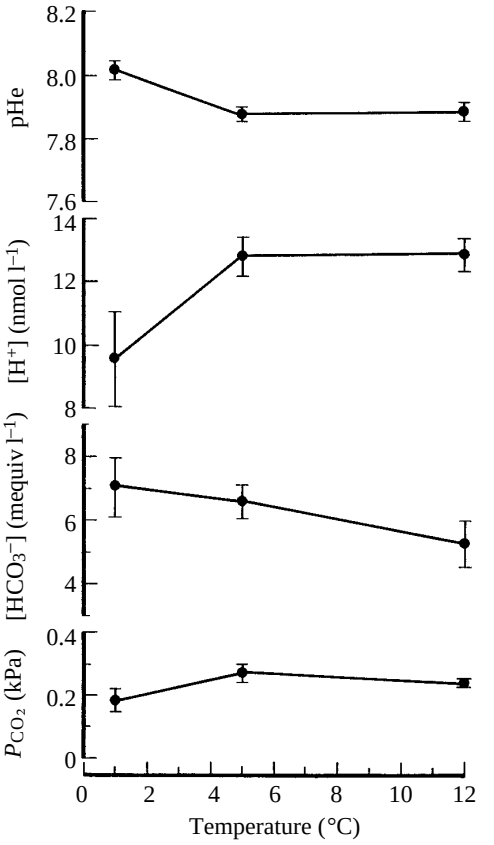

Fig. 1. Mean acid–base values (± S.E.M., N=5–8) in the postbranchial haemolymph of winter crayfish held at 1, 5 or 12 ˚C. Variables include direct measurements of extracellular pH (pHe) and calculated H^+ concentrations ($[H^+]$), bicarbonate levels ($[HCO_3^-]$) and CO_2 partial pressures (P_{CO_2}).

but the mean values at both these temperatures were statistically similar, being 0.19±0.03 kPa (N=5) at 1 ˚C and 0.24±0.03 kPa (N=8) at 12 ˚C. However, there was a significant rise in P_{CO_2} between 1 and 5 ˚C. At 5 ˚C, mean P_{CO_2} was 0.29±0.05 kPa (N=7), but there was no change in mean P_{CO_2}

Table 2. *Mean values for pHe, total CO_2 and* P_{CO_2} *measured* in vitro *at 1 °C for haemolymph withdrawn from animals held at 1, 5 and 12 °C, together with changes in pHe, H^+ concentration and* P_{CO_2} *values between 1 and 12 °C*

Temperature group (°C)	pHe	Total CO_2 (mmol l^{-1})	P_{CO_2} (kPa)	ΔpH	Δ$[H^+]$ (nmol l^{-1})	ΔP_{CO_2} (kPa)	N
In vitro							
1	7.80±0.02	4.00±0.62	0.17±0.02	–0.13±0.04	+5.3±2.2	+0.11±0.03	4
5	7.99±0.01	6.49±0.78	0.18±0.02	–0.16±0.04	+4.6±1.2	+0.16±0.04	5
12	8.11±0.03	7.06±0.55	0.16±0.01	–0.23±0.01	+4.8±0.1	+0.13±0.04	5
In vivo							
1	8.02±0.03	7.16±0.92	0.19±0.04	–0.12	+3.3	+0.05	5

Values are means ± S.E.M.
Values are compared with *in vivo* values obtained from animals held at 1 °C.
N, represents number of observations.

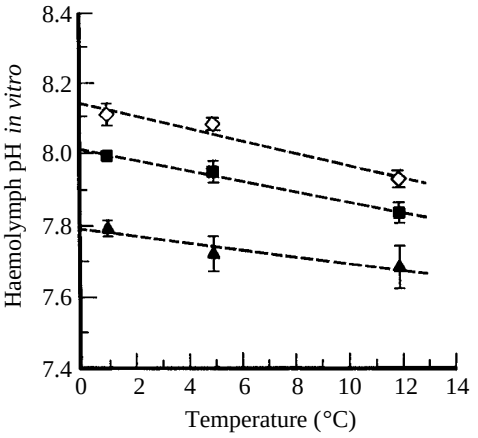

Fig. 2. Closed-system changes in haemolymph pHe with increasing temperature *in vitro*. Values are given ± S.E.M. (N=4–5) and represent haemolymph samples withdrawn from crayfish held at 1 °C (▲), 5 °C (■) or 12 °C (◇). The broken lines describe the relationships between temperature and pHe determined by linear regression analysis. The regression equations are as follows: pH=7.79−0.010t (r^2=−0.50) for animals held at 1 °C, pH=8.02−0.015t (r^2=−0.85) for animals held at 5 °C and pH=8.14−0.016t (r^2=−0.82) for animals held at 12 °C.

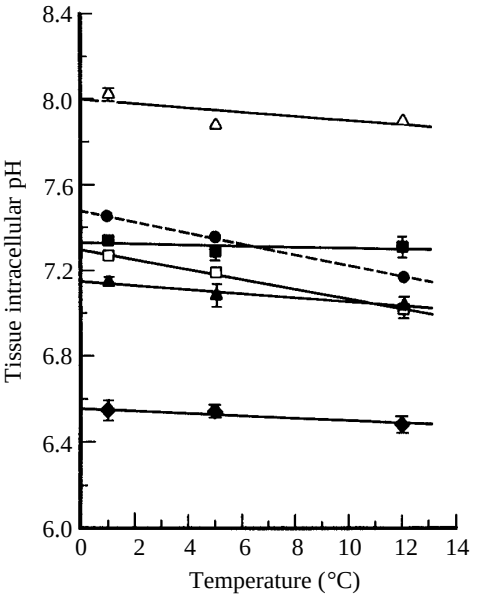

Fig. 3. The relationship between temperature and intracellular pH in the heart (▲), claw muscle (■), abdominal muscle (□) and hepatopancreas (◆) from animals held at 1, 5 or 12 °C. The individual relationships between pHi and temperature and between $[H^+]$ and temperature, expressed respectively as pH units °C^{-1} and an increase in $[H^+]$ between 1 and 12 °C, are as follows: heart (−0.010; +23.8 nmol l^{-1}), claw muscle (−0.003; +8.4 nmol l^{-1}), abdominal muscle (−0.025; +47.8 nmol l^{-1}) and hepatopancreas (−0.006; +44.0 nmol l^{-1}). Temperature-related changes in pN at −0.019 pH units °C^{-1} (●, broken line), and *in vivo* measurements of pHe at −0.009 pH units °C^{-1} (△) with an $[H^+]$ value of +3.3 nmol l^{-1} between 1 and 12 °C, are also included on this diagram. Values are means ± S.E.M., N=5–9.

between 5 and 12 °C, mirroring the changes in haemolymph pH values with temperature (Fig. 1). Mean values for lactate concentrations in the haemolymph were 0.24±0.06 mmol l^{-1} (N=5) at 1 °C, 0.32±0.16 mmol l^{-1} (N=7) at 5 °C and 0.17±0.07 mmol l^{-1} (N=8) at 12 °C. These values did not differ significantly from each other.

In vitro *experiments*

Changes in pHe with temperature, in haemolymph samples withdrawn from crayfish held at 1, 5 and 12 °C and subjected to an increase in temperature *in vitro*, are illustrated in Fig. 2 and associated variables are listed in Table 2. At 1 °C, mean pHe was 0.19 units lower in the 1 °C group than in the 5 °C group, and 0.31 units lower than the values obtained from the 12 °C group. The C_{CO_2} (Table 2) and HCO_3^- values obtained in animals held at 1 °C were significantly lower than those obtained in animals held at 5 and 12 °C, but P_{CO_2} levels were not significantly different from the values obtained in the other two groups, regardless of *in vitro* temperature.

The variation in pHe with temperature in haemolymph taken from animals at 1 °C was −0.010 pH units °C^{-1}. In contrast, pHe of haemolymph taken from animals held at 5 °C varied with temperature by −0.015 pH units °C^{-1} and pHe of haemolymph taken from animals acclimated to 12 °C varied by −0.016 pH units °C^{-1}. However, values for $[H^+]$ between 1 and 12 °C were similar for all three groups of animals (Table 2). Consistent with closed-system conditions, crayfish haemolymph from each temperature group showed no significant change in C_{CO_2} and calculated bicarbonate levels *in vitro* with increasing temperature, but there was a significant increase in calculated P_{CO_2} levels between 1 and 12 °C in all three temperature groups (Table 2).

Intracellular acid–base status

Table 1 displays the values obtained for tissue water content of heart, hepatopancreas, abdominal and claw muscle required for the correction of intracellular pH following the equations given by Pörtner *et al.* (1990). As was found by Gaillard and Malan (1983), tissue water content values were independent of temperature.

The relationships between pHi and temperature in heart, hepatopancreas, abdominal and claw muscle are shown in Fig. 3. At 5 °C, mean pHi was the most alkaline in claw muscle (7.27±0.07, N=5), being progressively more acidic in the abdominal muscle (7.19±0.04, N=5), the heart (7.08±0.05, N=5) and finally the hepatopancreas (6.58±0.05, N=5), whose pH was 1.30 units lower than mean pHe in the haemolymph. This variable, denoted as pHe-i, was 0.80 pH units for the heart, 0.69 pH units for the abdominal muscle and 0.61 pH units for the claw muscle.

With change in water temperature, there was no significant change in pHi of the claw muscle and hepatopancreas, giving respective temperature coefficients of $\Delta pHe/\Delta t$=−0.003 and −0.006 pH units °C^{-1} between 1 and 12 °C. Abdominal muscle pHi, however, decreased significantly between 1 and 12 °C,

Table 3. *Differences in the mean values for pHi, H^+ concentration and metabolic acid load in the heart, hepatopancreas, abdominal and claw muscles of crayfish between the acclimation temperatures of 1 and 12 °C*

Tissue	ΔpHi	Δ[H^+] (nmol l^{-1})	Δ[HCO_3^-] (mequiv l^{-1})	ΔH_m^+ (mmol kg^{-1})
Heart	–0.12	23.8	–0.24	1.80
Abdominal muscle	–0.27	47.8	–0.58	6.88
Claw muscle	–0.05	8.4	–0.19	0.92
Hepatopancreas	–0.06	44.0	–0.04	–

ΔH_m^+, metabolic acid load.

resulting in a $\Delta pHe/\Delta t$ value of -0.025 pH units $^\circ C^{-1}$, while mean pHi of heart muscle also dropped significantly with a gradient of -0.010 pH units $^\circ C^{-1}$. In contrast, intracellular H^+ concentrations increased to an equal extent in both the abdominal muscle and the hepatopancreas, to a lesser extent in the heart and much less in the claw muscle, between 1 and 12 °C (Table 3).

Mean intracellular [HCO_3^-] levels at 1 °C, calculated using extracellular P_{CO_2} and pK_1' values, were highest in the claw muscle (1.45±0.07 mequiv l^{-1}, N=5), relatively lower in the abdominal muscle (1.22±0.03 mequiv l^{-1}, N=5) and the heart (0.91±0.05 mequiv l^{-1}, N=5) and lowest in the hepatopancreas (0.21±0.03 mequiv l^{-1}). In general, there was a gradual decline in [HCO_3^-] in all tissues with increasing temperature; the decline was greatest in the abdominal muscle, then the heart, the claw muscle and finally the hepatopancreas. The falls in calculated [HCO_3^-] in the latter two tissues were not significant between 1 and 12 °C (Table 3). Consequently, the claw muscle continued to display the highest mean [HCO_3^-] at 12 °C (1.22±0.12 mequiv l^{-1}, N=9), with mean values in the heart and abdominal muscle falling to 0.65±0.06 mequiv l^{-1} (N=6) and 0.61±0.05 mequiv l^{-1} (N=6) at 12 °C, respectively. Bicarbonate levels in the hepatopancreas were 0.17±0.01 mequiv l^{-1} at 12 °C.

The apparent base deficit observed in the various muscle tissues with increasing temperature was accompanied by an increase in H^+ concentration at relatively constant P_{CO_2}, indicating a metabolic acidosis, especially in the abdominal muscle where the increase in [H^+] was highly significant. The resultant values for metabolic acid load calculated in the heart, abdominal and claw muscles between 1 and 12 °C are given in Table 3. The metabolic acid load in the abdominal muscle was 3.8 times larger than the acid load in the heart and 7.5 times larger than the value calculated for claw muscle.

Discussion

Extracellular acid–base balance

Comparisons of the haemolymph pH data collected in this study from temperature-acclimated winter crayfish with values obtained from temperature-acclimatised crayfish during the winter are given in Fig. 4. This figure is adapted from Quist *et al.* (1977) and shows the relationship between temperature and blood pH in a range of poikilotherms. The predicted limits of the pH range are based on the assumption that a constant relative alkalinity is maintained. The pH values obtained in this study from *Austropotamobius pallipes* at 5 °C were all at the low end or lower than the predicted range of values given at the same temperature in Fig. 4. Mean haemolymph pH at 1 °C was also at the lower limit of the predicted pH range, while values at 12 °C were within this range, stressing that mean haemolymph pH values obtained at 1 and 5 °C were relatively acidotic compared with the values obtained by extrapolation from data for other poikilothermic animals. This is reflected in the depressed $\Delta pHe/\Delta t$ coefficient of -0.009 pH units $^\circ C^{-1}$, which is lower than most coefficients reported in the literature for other aquatic decapod crustaceans (see reviews by Cameron, 1986; Truchot, 1987) and about half the $\Delta pN/\Delta t$ value of -0.019 pH units $^\circ C^{-1}$ calculated between 1 and 12 °C. The changes in alkalinity ratio with acclimation temperature and the depressed $\Delta pHe/\Delta t$ value indicate that winter crayfish acclimated to 1 and 12 °C did not maintain a constant relative alkalinity in the extracellular compartment, deviating from alphastat regulation. In addition, calculation of corresponding H^+ concentrations in the haemolymph gave an [H^+] value of +3.3 nmol l^{-1}, which is less than the predicted value of +4.12 nmol l^{-1} obtained from the extrapolation of 12 °C values back to 1 °C, assuming a $\Delta pHe/\Delta t$ value of -0.019 pH units $^\circ C^{-1}$ (i.e. a constant relative alkalinity). In the wild population during a winter season, haemolymph pH

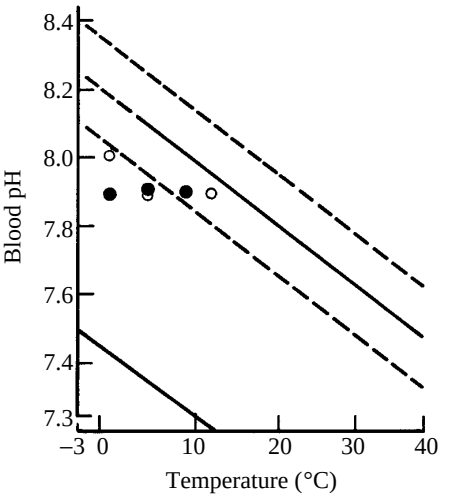

Fig. 4. Modification of a diagram taken from Quist *et al.* (1977) to show limits for the pH range predicted by Rahn (1966) and Garey (1972) for poikilotherms that maintain a constant relative alkalinity in the blood with change in temperature down to −3 °C. The symbols superimposed on this diagram represent haemolymph pH values of seasonally acclimatised crayfish in the wild population (●) obtained by Whiteley and Taylor (1993) and of crayfish acclimated to temperature changes in the laboratory (○) obtained in this study.

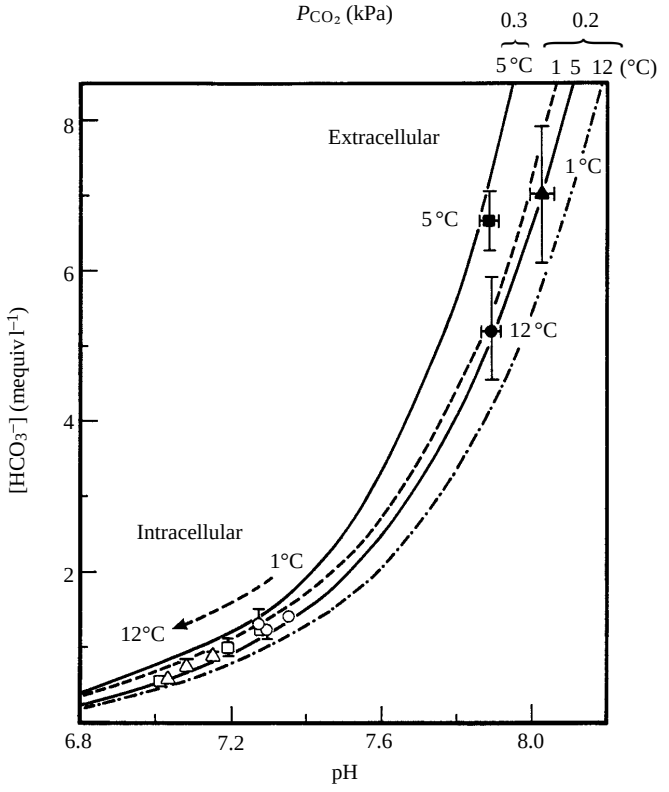

Fig. 5. A pH–bicarbonate diagram to show the relationship between the main acid–base variables in both the extracellular and intracellular compartments of crayfish held at the three different acclimation temperatures. The filled symbols represent mean values for extracellular acid–base status, and the open symbols represent mean intracellular acid–base values in the claw muscle (○), abdominal muscle (□) and heart (△). The two continuous lines show the positions of the 0.2 and 0.3 kPa P_{CO_2} isopleths calculated at 5 °C using appropriate pK_1' and αCO_2 constants. The changing positions of the 0.2 kPa P_{CO_2} isopleth due to temperature-related changes in physico-chemical constants are also included. In the haemolymph between 5 and 1 °C, there was a combined respiratory and metabolic alkalosis, but between 5 and 12 °C there was a potential metabolic acidosis, masked by shifts in the P_{CO_2} isopleths, so there was no change in pHe. In the abdominal muscle and the heart, pHi decreased with rising temperature with smaller changes in the positioning of the 0.2 kPa P_{CO_2} isopleth, resulting in a metabolic acidosis in both tissues between 1 and 12 °C. In contrast, the pHi of the claw muscle (○) decreased between 1 and 5 °C, owing to a slight metabolic acidosis, but remained unchanged between 5 and 12 °C. Mean values are given ± S.E.M. (N=5–9) with the smaller standard errors being contained within the symbols.

remained unchanged at a value of about 7.9 at all environmental temperatures between 1 and 11 °C (Fig. 4). The small change in pHe and $[H^+]$ with temperature measured in the laboratory population of crayfish could be due to incomplete acclimation to temperature reduction, which may never equal seasonal acclimatisation.

Changes in pHe between 1 and 12 °C were accompanied by a fall in HCO_3^- ($\Delta HCO_3^- = -1.8$ mequiv l^{-1}) and a slight increase in P_{CO_2} (P_{CO_2}=0.05 kPa), as expected in aquatic poikilotherms in response to a rise in temperature (Howell *et al.* 1973). The response differed, however, depending on the direction of the temperature change from the *in situ* temperature of 5 °C. Between 5 and 1 °C, pHe increased, whereas it remained unchanged between 5 and 12 °C. The relationship between pHe and associated changes in P_{CO_2} and HCO_3^- are summarised in the pH–bicarbonate diagram (Fig. 5). Closer examination of the mean extracellular acid–base variables at each temperature on this diagram shows that crayfish cooled from 5 to 1 °C experienced a combined respiratory and metabolic alkalosis, whereas the animals warmed to 12 °C experienced a potential metabolic acidosis. Overall, the potential effects of the measured alterations in P_{CO_2} and HCO_3^- levels on haemolymph pH were diminished as a result of the temperature-related shifts in the P_{CO_2} isopleths, as outlined on Fig. 5. The resultant small $\Delta pHe/\Delta t$ value is likely to be a result of active regulation of pHe within narrow limits by regulation of P_{CO_2} and HCO_3^- levels in the haemolymph as temperature changes.

The relationship between extracellular acid–base status and temperature was investigated further by following changes in pHe resulting from passive changes in physico-chemical constants in a closed system. Haemolymph withdrawn from crayfish held at 1, 5 and 12 °C showed a decline in pHe at constant total CO_2 as temperature increased as a result of an increase in P_{CO_2}, which is typical in a closed system (Truchot, 1973, 1978; Gaillard and Malan, 1985). Haemolymph withdrawn from animals held at 5 and 12 °C followed similar temperature coefficents (−0.015 and −0.016 pH units $°C^{-1}$, respectively) to those obtained in the haemolymph of *Astacus leptodactylus* (−0.0168 and −0.0164 pH units $°C^{-1}$; Dejours and Armand, 1983; Gaillard and Malan, 1985). The haemolymph samples withdrawn from the 1 °C group, however, were characterised by a shallow $\Delta pHe/\Delta t$ gradient over a relatively lower range of pH and HCO_3^- values. The initial *in vitro* $[HCO_3^-]$ value for the pooled haemolymph sample from animals at 1 °C was approximately 4 mequiv l^{-1}, which is 3 mequiv l^{-1} lower than the value measured *in vivo* at 1 °C. This discrepancy between *in vitro* and *in vivo* HCO_3^- levels suggests either loss of HCO_3^- or addition of H^+ during sampling and storage of the haemolymph samples. These differences between haemolymph samples withdrawn from animals held at 5 and 12 °C and those held at 1 °C cannot be explained and clearly need further investigation. However, the present study emphasizes the relative changes in acid–base status induced by warming the samples to 12 °C and it is interesting to note that the temperature-related changes in $[H^+]$ in the 1 °C group result in similar $[H^+]$ values between 1 and 12 °C to those obtained in haemolymph samples withdrawn from animals held at 5 and 12 °C (Table 2). The different $\Delta pHe/\Delta t$ values are due to the inverse logarithmic relationship between pH and $[H^+]$. Thus, under closed-system conditions, although $\Delta pHe/\Delta t$ varied with the absolute pH, similar $[H^+]$ values were obtained from all three groups of crayfish and

these matched the value predicted following a constant relative alkalinity *in vivo*. Therefore, in closed-system conditions, haemolymph pH/temperature relationships in *A. pallipes* during the winter were similar to those observed in aquatic crustaceans that maintain a constant relative alkalinity with change in temperature.

Intracellular acid–base status

Intracellular pH data for crustacean tissues at 5 and 1 °C are currently not available for comparison with the present results, but intracellular pH values in the heart and abdominal muscles at 12 °C were generally found to be lower than corresponding values obtained in *Astacus leptodactylus* (Gaillard and Malan, 1983), *Pacifasticus leniusculus* (Wheatly *et al.* 1991) and *Callinectes sapidus* (Wood and Cameron, 1985) as determined by the distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO). For example, heart pHi at 12 °C in the present study was 7.03 compared with 7.41 at 13 °C in *A. leptodactylus* and 7.66 at 12 °C in *P. leniusculus*. The DMO technique may overestimate pHi by partitioning into the mitochondria (H.-O. Pörtner, personal communicaton). Measurements of pHi by ^{31}P nuclear magnetic resonance spectroscopy of the abdominal muscle in the prawn *Palaemon serratus* during the winter by Thebault and Raffin (1991) gave a pHi value of 7.13±0.04 at 7 °C, which was lower than the value of 7.19±0.04 obtained in the same tissue in winter crayfish at 5 °C. Values for pHi in the claw muscle were similar, irrespective of the measuring technique used, with a value of 7.30 obtained in this study and a value of 7.32 obtained in *A. leptodactylus* using DMO (Gaillard and Malan, 1983). Clearly, there are discrepancies between the pHi values obtained from crustaceans depending on the measurement technique used and the tissues studied, with the homogenising technique generally giving lower pHi values than the DMO technique. Consequently, the values arising from the present study can be most usefully considered relative to one another rather than compared with the small number of previous studies.

All tissues were more acidic than the haemolymph, with claw muscle being the most alkaline of the tissues (pHe-i at 12 °C was 0.60), followed by abdominal muscle (pHe-i at 12 °C was 0.87), heart (pHe-i at 12 °C was 0.89) and finally hepatopancreas, which was the most acidic of the tissues, at 1.42 pH units lower than the haemolymph at 12 °C. The pHe-i values corresponded to differences in intracellular HCO_3^- levels, with the most alkaline compartment (the claw muscle) having the highest HCO_3^- levels.

With change in acclimation temperature there was hardly any change in pHi of the claw muscle and the hepatopancreas. The resulting temperature coefficient for claw muscle (-0.003 pH units °C^{-1}) was lower than any of the coefficients obtained for slow aerobic muscle in poikilothermic animals (Heisler, 1984) but similar to the value of -0.005 pH units °C^{-1} obtained in the red muscle of winter rainbow trout *Oncorhynchus mykiss* (S. Taylor, personal communication), suggesting a seasonal component to pHi regulation in aerobic tissues. In winter crayfish, heart pHi varied in parallel to the changes in haemolymph pH (ΔpHi/Δt=-0.010 pH units °C^{-1}), indicating a close association between intracellular and extracellular acid–base balance in the myocardium. As this tissue is thin-walled and in intimate contact with the haemolymph, it is likely to maintain a stable relationship. The pHi of the abdominal muscle had a steeper temperature coefficient of -0.025 pH units °C^{-1}, showing the closest agreement between ΔpHi/Δt and the ΔpN/Δt value of -0.019 pH units °C^{-1} between 1 and 12 °C (see Fig. 3). Conversion of pHi to corresponding [H^+] values revealed that H^+ accumulated in the tissues to different extents in response to the rise in temperature. In general, [H^+] increased with increase in temperature as a result of a metabolic acid load that was most extensive in the abdominal muscle. Interestingly, the increase in [H^+] between 1 and 12 °C in the hepatopancreas was equivalent to the change obtained in the abdominal muscle despite its low ΔpHe/Δt value. This was due to the logarithmic relationship between pH and [H^+], with similar changes in [H^+] causing smaller changes in pHi at the lower physiological pHi values found in the hepatopancreas, as discussed with reference to pHe values above. Calculation of the metabolic acid load in the hepatopancreas was not performed because of the lack of information on buffering capacity in this tissue.

The differences in acid–base response to changes in temperature over the range 1–12 °C between cardiac, claw and abdominal muscles indicate different mechanisms of pHi regulation, which may be related to the relative functional and physiological roles of each muscle group, particularly during the winter months. The fast glycolytic muscle fibres of the deep abdominal flexor muscle are more likely to accumulate a metabolic acid load with increase in temperature due to an increase in lactic acid production than are the slow aerobic fibres of the heart and claw. The accumulation of a larger metabolic acid load in the abdominal muscle may also reflect the relatively poor perfusion of this primarily anaerobic tissue. In effect, this tissue could be acting like a functionally closed system in the short term, with intracellular pH varying with temperature relatively independently from the extracellular fluid. In contrast, the small change in H^+_m observed in the claw muscle indicates active regulation of H^+ to maintain a set pH value irrespective of changes in temperature or pHe. The kinetics of pHi regulation may vary between tissues, as suggested by Rodeau (1984), who found slower kinetics for pHi regulation in the extensor muscle of the leg compared with nervous tissue in the crayfish.

The mechanisms regulating temperature-related changes in pHi and [H^+] cannot be deduced from these results, but may include changes in the rates of production or efflux of protons or of specific buffering capacity, as discussed by Gaillard and Malan (1985) and Wood and Cameron (1985). Variations in ΔpHi/Δt between the tissues indicate differences in the relative importance of the active mechanisms controlling pHi or in the predominance of various protein buffers with differing pK values (Cameron, 1989).

Irrespective of the regulatory mechanisms involved, the maintenance of a set pHi value in the claw muscle may serve to reduce rates of intermediary metabolism in this tissue during the winter months in order to preserve glycogen stores when resources are low, as suggested by Johnston *et al.* (1983) and Cameron (1989). Such a response was recorded in the American eel (*Anguilla rostrata*), which enters a state of torpor in the winter and is characterised by relatively acidic pHi values in the red and white muscle, resulting in a depressed $\Delta\mathrm{pHi}/\Delta t$ coefficient of $-0.008\,\mathrm{pH\,units\,^{\circ}C^{-1}}$ (Walsh and Moon, 1982). These authors suggested that the skeletal muscles were relatively inactive at low temperatures, with a reduction in metabolic rate brought about by the presence of suboptimal pH values for enzyme function. These animals also showed organ-specific deviations from alphastat regulation: the $\Delta\mathrm{pHi}/\Delta t$ in the heart and liver followed the classic relationship, possibly maintaining the metabolic rate over the full range of temperatures. Different patterns of metabolic regulation were also observed in these winter crayfish: the $\Delta\mathrm{pHe}/\Delta t$ value obtained in the abdominal muscle, in constrast to that in the claw muscle, followed a constant relative alkalinity, exhibiting alphastat regulation. This may maintain optimal conditions for enzyme function in this fast glycolytic muscle to ensure effective 'tail flick' responses, enabling escape from predators at low winter temperatures.

In summary, the present study of winter crayfish acclimatised to 5 °C in their natural environment and acclimated to 1 and 12 °C revealed that, although $\Delta\mathrm{pHe}/\Delta t$ maintained constant relative alkalinity *in vitro*, as a result of passive changes in physico-chemical constants, *in vivo* it did not do so because of a relative acidosis at 5 and 1 °C. In general, intracellular pH regulation was independent of both temperature and extracellular pH and each tissue operated independently, as shown in a number of other studies on pHi regulation in aquatic crustaceans (Gaillard and Malan, 1983; Tyler-Jones and Taylor, 1988; Wheatly *et al.* 1991) and in a number of aquatic poikilothermic vertebrates (Boutilier *et al.* 1987; Heisler *et al.* 1976; Heisler, 1984, 1986). The variation in $\Delta\mathrm{pHi}/\Delta t$ values observed in the tissues could be related to differential regulation of metabolism during the winter months, with a reduction in energy usage in relatively inactive tissues at low temperatures, but maintenance of functions essential for survival, such as the 'tail flick' responses of the abdominal muscles.

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