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TEMPERATURE EFFECTS ON HAEMOLYMPH ACID–BASE STATUS *IN VIVO* AND *IN VITRO* IN THE TWO-STRIPED GRASSHOPPER *MELANOPLUS BIVITTATUS*

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Summary

In this study, I examine the effect of temperature on haemolymph acid–base status *in vivo* and *in vitro* in the two-striped grasshopper *Melanoplus bivittatus*. *Melanoplus bivittatus* experience wide (up to 40°C) diurnal body temperature fluctuations in the field, but maintain body temperature relatively constant during sunny days by behavioural thermoregulation. Haemolymph pH was statistically constant (7·12) between 10 and 25°C, but decreased by −0·017 units °C^{-1} from 25 to 40°C. Relative alkalinity and fractional protein dissociation were conserved only at body temperatures at which feeding and locomotory activity occur, above 20°C. Haemolymph total CO_2 (C_{tot}) increased from 10 to 20°C and decreased from 20 to 40°C. Haemolymph P_{CO_2} increased from 10 to 20°C and was statistically constant between 20 and 40°C. Carbonic acid pK_{app} in haemolymph was 6·122 at 35°C, and decreased with temperature by −0·0081 units °C^{-1}. Haemolymph buffer value averaged −35 mequiv l^{-1} pH unit^{-1}. Haemolymph pH changes with temperature were small (less than −0·004 units °C^{-1}) *in vitro* at constant P_{CO_2}. Therefore, passive physicochemical effects cannot account for the pattern of acid–base regulation *in vivo*. The temperature shift from 10 to 20°C was accompanied by a net addition of 4·2–6·2 mmol l^{-1} of bicarbonate equivalents to the haemolymph. The temperature shift from 20 to 40°C was accompanied by a net removal of 10–14 mmol l^{-1} of bicarbonate equivalents from the haemolymph. Haemolymph acid–base regulation *in vivo* during temperature changes is dominated by active variation of bicarbonate equivalents rather than by changes in P_{CO_2} as observed for most other air-breathers.

Introduction

In the majority of poikilothermic animals that have been studied, increasing temperature is accompanied by decreasing extracellular pH (reviewed by Reeves, 1977; Heisler, 1984; Glass *et al.* 1985). This effect has been attributed to the maintenance of (1) constant fractional dissociation of imidazole groups or

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'alphastat regulation' (Reeves, 1977) or (2) constant relative alkalinity (Howell *et al.* 1970). In many organisms the slope of the line describing the decrease in pH with temperature is significantly below that compatible with either of these two hypotheses (Heisler, 1984; Glass *et al.* 1985), and the physiological significance of pH changes with temperature remains controversial. Examination of pH regulation with temperature in diverse taxa is an important test of these hypotheses, as pH and temperature effects on enzyme action (Wilson, 1977*a*,*b*) should apply to all organisms. In this study I examined the effect of body temperature on haemolymph acid–base status in the two-striped grasshopper *Melanoplus bivittatus* (Say).

Insects are the largest and most diverse ectothermic taxon, and are important models for testing the significance of pH regulatory patterns with temperature. Only one study has examined the relationship of pH to body temperature in insects. Storey *et al.* (1984) used P-NMR data to examine temperature effects on intra- and extracellular pH in groups of freeze-tolerant gall fly larvae from −12 to 15°C. In gall fly larvae, haemolymph pH remains constant while intracellular pH varies as predicted by alphastat regulation, maintaining relative alkalinity and calculated protein dissociation fraction at all temperatures.

Two general mechanisms have been proposed for the regulation of extracellular pH with temperature in animals. In most air-breathing animals, the decrease of extracellular pH with temperature is caused by increasing P_{CO_2} due to reduced ventilation relative to metabolic CO_2 production (reviewed by Jackson, 1982). Gill-breathers such as fish and crustaceans, however, often accomplish extracellular pH regulation by transmembrane ion movements (reviewed by Truchot, 1978; Heisler, 1984). *A priori*, one might expect air-breathing insects such as grasshoppers to utilize variation of P_{CO_2} for the control of haemolymph pH with temperature.

Grasshoppers thermoregulate behaviourally when possible but, nonetheless, may experience substantial diurnal body temperature variation (Uvarov, 1977), making them excellent insects for testing the effect of temperature on pH regulation. One objective of this study was to examine diurnal changes of body temperature in the field for *M. bivittatus*, and to relate these data to the pattern of acid–base regulation. To aid in distinguishing among possible haemolymph pH regulatory mechanisms in this grasshopper, I also examined CO_2 solubility, carbonic acid pK_{app}, haemolymph buffering, and the effect of temperature on pH and $[HCO_3^-]$ *in vitro* at constant P_{CO_2}.

Materials and methods

Animals

Melanoplus bivittatus were collected from a disturbed grassland site near Lafayette, CO, and were kept in the laboratory for 1–3 weeks before testing. Only adult females were used in the haemolymph studies because of their larger size. Animals were kept in screen cages with a 14 h:10 h light:dark cycle, with a

photothermal gradient during the light cycle to allow behavioural thermoregulation. Temperatures within cages varied from 30 to 45 °C during the light cycle and averaged 23 °C at night. Relative humidity averaged 30 %. Animals were fed on wheat sprouts and wheat bran treated with Tri-Sulfa-G (Norden Laboratories) to control amoebic infections. Under these conditions animals lived for 5–7 weeks as adults, and laid numerous eggs.

Field body temperature measurement

Observations of field behaviour and diurnal body temperature variations were conducted at the collection site near Lafayette, CO. Observations were made between 05.00 and 20.00 h over 6 days between 25 August and 25 September 1986, during the peak of local abundance of this species. Thoracic temperature (T_{th}) was measured with a copper–constantan thermocouple threaded through a 26 gauge needle, connected to a Wescor TH-65 thermocouple thermometer. Ambient temperature (T_a) was measured with a second thermocouple in the shade, 6 cm above ground level. For T_{th} measurement, grasshoppers were captured by hand and held with a cloth while the thermocouple was inserted into the centre of the thorax. Time between animal capture and T_{th} measurement averaged 10 s, and ranged from 5 to 15 s. Animals measured in less than 8 s did not differ in temperature from those measured in over 12 s.

Haemolymph acid–base status in vitro

CO_2 solubility coefficient (α)

Physical CO_2 solubility was measured in 0·01 mol l^{-1} HCl (α_{HCl}) and in a 1:1 mix of 0·01 mol l^{-1} HCl and *M. bivittatus* serum (α_{mix}). Haemolymph from 8–12 animals was pooled, allowed to clot at room temperature, and then centrifuged at about 1400 *g* for 10 min. The mix of haemolymph supernatant and dilute acid had a pH below 3, ensuring that no HCO_3^- was present. 100 μl droplets were equilibrated for 1–2 h with water-saturated 20 % CO_2 (balance N_2). Haemolymph was sampled from the drop with a Hamilton syringe (100 μl), flushed with the equilibration gas. Total CO_2 (C_{tot}, mmol l^{-1}) was measured on 20 μl samples by injecting the fluid into a 250 μl glass syringe filled with 0·01 mol l^{-1} HCl plus 0·1 % (w/v) octanol. The syringe was immediately sealed with mercury, and the fluid stirred with a small magnetic stir-bar for 40 s before injecting the syringe contents into the cuvette of a Radiometer BMS 3K2 blood gas analyser. The P_{CO_2} of the fluid was measured with the Radiometer P_{CO_2} electrode. The change in P_{CO_2} relative to background was linearly related to the C_{tot} of $NaHCO_3$ standards. The variability of duplicate samples was approximately ±0·5 mmol l^{-1}. The solubility coefficient (α, mmol l^{-1} $mmHg^{-1}$; 1 mmHg = 133·3 Pa) for each fluid was calculated using Henry's law:

$$\alpha = C_{tot}P_{CO_2}^{-1} . \quad (1)$$

The physical solubility of CO_2 in haemolymph (α_{haem}) was calculated from:

$$\alpha_{haem} = f_{haem}^{-1}(\alpha_{mix} - f_{HCl}\alpha_{HCl}), \quad (2)$$

where f_{haem} and f_{HCl} are the fractions of haemolymph and dilute HCl in the mix.

Carbonic acid apparent dissociation constant (pK_{app})

The apparent pK (pK_{app}) of carbonic acid in *M. bivittatus* haemolymph was measured at 10°C, 25°C and 35°C at 2 %, 5 %, 10 % and 18·7 % CO_2 (humidified, balance N_2) provided by Wösthoff gas-mixing pumps. Haemolymph was pooled on ice from 20–30 animals kept at room temperature, and centrifuged for 5 min at 1400 *g*. Total protein loss during the process was less than 5 %, as haematocrits were under 3 % and clotting was minimal if the haemolymph was kept on ice before centrifugation. Clotting in insect haemolymph requires factors from the haemocytes and the plasma; therefore separation of haemocytes from plasma prevents clotting, without alteration of the remaining plasma proteins (Brehelin, 1979). Haemolymph was frozen, and kept at −70°C for less than 1 week before equilibrations. Preliminary experiments indicated that freezing did not affect either the pH or the C_{tot} of haemolymph samples at a given P_{CO_2} and temperature.

Haemolymph melanizes quite rapidly in the presence of oxygen. To eliminate possible effects of this on acid–base status, haemolymph equilibration gases were oxygen-free. The concentration of oxygen in insect haemolymph does not exceed its physical solubility (Bishop, 1923; Babers, 1941), so complications due to a Haldane effect are unlikely. With no oxygen present, haemolymph exhibited a relatively constant decrease in pH with time (roughly $-0{\cdot}06$ pH units h^{-1}) which was arrested by the addition of 20 mmol l^{-1} NaF. Under these conditions, haemolymph pH was stable for at least 8 h. Droplets (100 μl) of haemolymph were equilibrated with 2 %, 5 %, 10 % or 18·7 % CO_2 (balance N_2) for at least 1 h and pH and C_{tot} were measured.

C_{tot} was measured as described above, except that samples were collected with polyethylene tubing (PE 10) filled with 0·9 % NaCl connected to a Hamilton syringe. Approximately 15 μl of haemolymph was drawn into the tubing, 2 μl was discarded and 10 μl was injected into the acid-filled syringe, as described above. Preliminary experiments using saline containing [^{14}C]lactate indicated that haemolymph sampled in this way was substantially diluted with saline (approximately 20 %). However, this dilution factor was relatively constant (S.D. = 3 %). This technique minimized the volume of haemolymph required and, after correcting for dilution, calculated pK_{app} values compared favourably with values in the literature (see Results).

Haemolymph pH was measured on 15 μl samples collected with glass microcapillary tubes. Samples were immediately transferred to the capillary electrode of a Radiometer BMS MK2 blood gas analyser thermostatted to the equilibration temperature. Samples were drawn into the pH electrode and followed by a solution of filtered, saturated KCl which filled the polyethylene transfer tube and provided an electrical bridge to the reference electrode. This technique yielded slightly different results from those if the polyethylene bridge was completely filled

with sample, so the electrode was calibrated with 15 μl samples of pH precision buffers (Radiometer S1500 and S1510) followed with saturated KCl, after repeated rinsing of the electrode with calibration buffer. When the pH of 15 μl haemolymph samples was measured after calibration with 15 μl of calibration buffers, the pH equalled that measured for 40 μl haemolymph samples measured after calibration with excess calibration buffers.

Temperature effects on haemolymph acid–base status at constant P_{CO_2}

Haemolymph from 30–40 animals kept at room temperature was pooled on ice. Haemolymph was centrifuged as described above, and the supernatant split into 100 μl samples, which were frozen at −70°C. Samples of this haemolymph were tonometered as described above at 2 %, 5 %, 10 % and 18·7 % CO_2 at 10°C, 25°C and 35°C, and pH and C_{tot} were measured. Tonometry was performed within 1 week of freezing.

Temperature effects on haemolymph acid–base status in vivo

For measurements of temperature effects on haemolymph acid–base status *in vivo*, animals were kept individually in cardboard cartons at test temperatures for 12–16 h. Preliminary studies showed that haemolymph pH values of animals kept for 1·5 and 16 h at a temperature were identical, suggesting that this time was sufficient for attainment of steady-state conditions. No food was provided, but each carton contained a moistened paper towel to prevent dehydration of the grasshopper. Haemolymph samples were obtained by grasping the animal and making a quick incision in the ventral neck membrane, and sampling from the haemolymph which appeared on the neck. Haemolymph pH and C_{tot} were measured as described above for pK_{app} experiments. Owing to haemolymph volume limitations, pH and C_{tot} were measured on separate animals. P_{CO_2} was calculated from the average values for pH and C_{tot} at each temperature.

Fractional imidazole dissociation was calculated according to Reeves (1976). Relative alkalinity was calculated from the K_w for water (Weast, 1977). Haemolymph protein was measured on 10 μl samples, diluted 9:1 (w/v) with water, and kept frozen at −70°C until analysis. Haemolymph protein was measured with a biuret reagent (Layne, 1957), using bovine serum albumin as a standard. Haemolymph total ammonia was measured spectrophotometrically on 50 μl samples, using a specific enzymatic assay (Sigma, kit 170-UV) and a Gilford model 260 spectrophotometer. Background rates of NADH oxidation in haemolymph without glutamate dehydrogenase were subtracted from measured absorbance changes. Samples were kept on ice for less than 1 h before measurement, as ammonia content was substantially elevated in frozen samples.

Results

Field body temperatures and behavioural thermoregulation

The distribution of grasshoppers during the 30 min before sunrise depended on

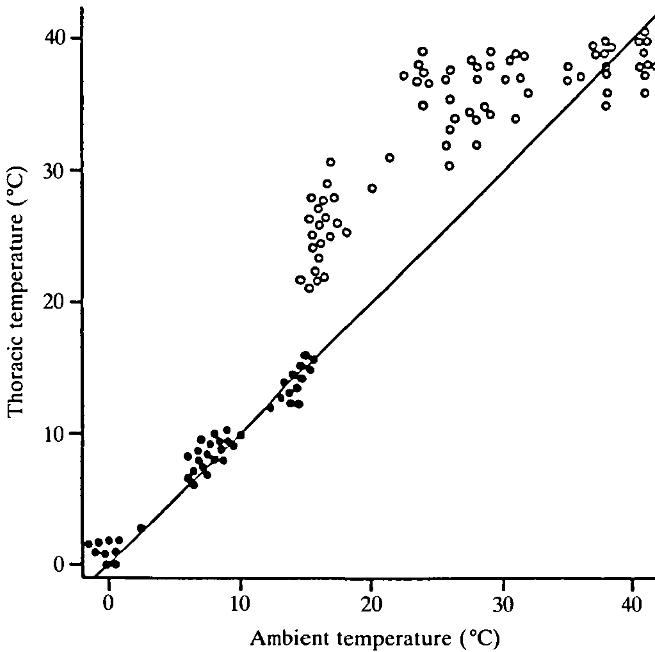

Fig. 1. Field body temperatures of *Melanoplus bivittatus*. Open circles represent thoracic temperatures measured after sunrise, closed circles those before sunrise. The diagonal line indicates equality of thoracic and ambient temperatures. Male and female thoracic temperatures did not differ ($P>0{\cdot}05$) and were pooled.

temperature. Below 5°C, grasshoppers were found on the ground under vegetation, with T_{th} at or just above T_a. At temperatures above 5°C, grasshoppers occupied upper regions of herbaceous vegetation, generally situated in sites which would become sunny microhabitats when the shrub was directly illuminated by the rising sun. In these animals, T_{th} was equal to or slightly lower than T_a (Fig. 1). After sunrise, at ambient temperatures between 15 and 35°C, grasshoppers were also generally located in the upper regions of vegetation. Body temperatures increased rapidly in the early morning hours as these animals elevated T_{th} above T_a behaviourally by choosing sunny microhabitats and basking. Above T_a values of 30°C, the slope of T_{th} on T_a was not significantly different from zero, indicating that these grasshoppers are excellent behavioural thermoregulators.

Temperature effects on haemolymph acid–base status in vitro

Haemolymph CO_2 solubility and pK_{app}

CO_2 solubility in *M. bivittatus* haemolymph and in $0{\cdot}01\,\mathrm{mol\,l^{-1}}$ HCl decreased with increasing temperature (Table 1). The CO_2 solubility coefficient in haemolymph (α_{haem}) was fitted to the following formula: $\alpha = 0{\cdot}1079 - 0{\cdot}020955 \ln T$ (°C); $r^2 = 0{\cdot}994$.

Carbonic acid pK_{app} in *M. bivittatus* haemolymph decreased with temperature,

Table 1. *The effect of temperature on CO_2 solubility coefficients in 10 mmol l^{-1} HCl and* Melanoplus bivittatus *haemolymph and the CO_2 pK_{app} in haemolymph*

	CO_2 solubility coefficients ($mmol\,l^{-1}\,mmHg^{-1}$)			
	10°C	20°C	30°C	40°C
0·01 mol l^{-1} HCl ($N=6$)	0·0668 ± 0·00032	0·0506 ± 0·00105	0·0380 ± 0·00086	0·0323 ± 0·00070
Melanoplus haemolymph ($N=6$)	0·0597 ± 0·00212	0·0452 ± 0·00247	0·0362 ± 0·00090	0·0309 ± 0·00133

	CO_2 pK_{app} in *Melanoplus* haemolymph		
	10°C	25°C	35°C
($N=10$)	6·324 ± 0·0132	6·210 ± 0·0260	6·122 ± 0·0157

Values are means ± S.E.

with a slope of −0·0081 pH units $°C^{-1}$ between 10 and 35°C (Table 1). There was no significant effect of haemolymph pH on pK_{app}.

Haemolymph buffer value and CO_2 capacitance

Plots of pH *vs* [HCO_3^-] were approximately linear from pH 7·1 to 6·65 (P_{CO_2}; 12–117 mmHg). Protein content averaged 45 mg ml^{-1} (S.E. = 0·89, $N=31$), and did not vary significantly in any of the *in vitro* tests. Buffer value, defined as $\Delta[HCO_3^-] \times \Delta pH^{-1}$, did not vary significantly with temperature, and averaged −34·9 mequiv l^{-1} pH $unit^{-1}$ (S.E. = 1·79, $N=31$). CO_2 combining curves were shifted downwards with increased temperature (Fig. 2). This was due to decreases in dissolved CO_2 and [HCO_3^-] at any given P_{CO_2}. Average CO_2 capacitance from 12 to 60 mmHg ($\Delta C_{tot} \times \Delta P_{CO_2}^{-1}$) did not vary with temperature, and averaged 0·200 mmol l^{-1} $mmHg^{-1}$ (S.E. = 0·0066, $N=9$).

Temperature effects on haemolymph pH and [HCO_3^-] in vitro *at constant P_{CO_2}*

Haemolymph pH tended to decrease slightly at all P_{CO_2} values from 10 to 25°C, with a slight trend towards a lesser temperature effect at higher P_{CO_2} values (Table 2). From 25 to 35°C, pH decreased at 2% CO_2 (−0·0039 pH units $°C^{-1}$), but it increased slightly at 10 and 18·7%. [HCO_3^-] changes with temperature showed a more complex pattern (Table 2). From 10 to 25°C, [HCO_3^-] decreased at all P_{CO_2} values, with the decrease being roughly proportional to P_{CO_2}. From 25 to 35°C, [HCO_3^-] decreased slightly at 2% CO_2, and increased at 10% and 18·7% CO_2. [$HCO_3^-{}_{(25)}$]/[$HCO_3^-{}_{(10)}$] was approximately 0·7 for all P_{CO_2} values. [$HCO_3^-{}_{(35)}$]/[$HCO_3^-{}_{(25)}$] was approximately 1·1 at 10 and 18·7% CO_2, and just under 1 at 2% CO_2. Changes in pH or [HCO_3^-] *in vitro* at constant P_{CO_2} were not evaluated for statistical significance owing to small sample size.

Temperature effects on haemolymph acid–base status in vivo

Haemolymph pH in *M. bivittatus* acclimated to test temperatures for 12–16h showed a biphasic pattern (Fig. 3). Haemolymph pH was statistically constant from 10 to 25°C. Above 25°C, pH decreased with increasing temperature

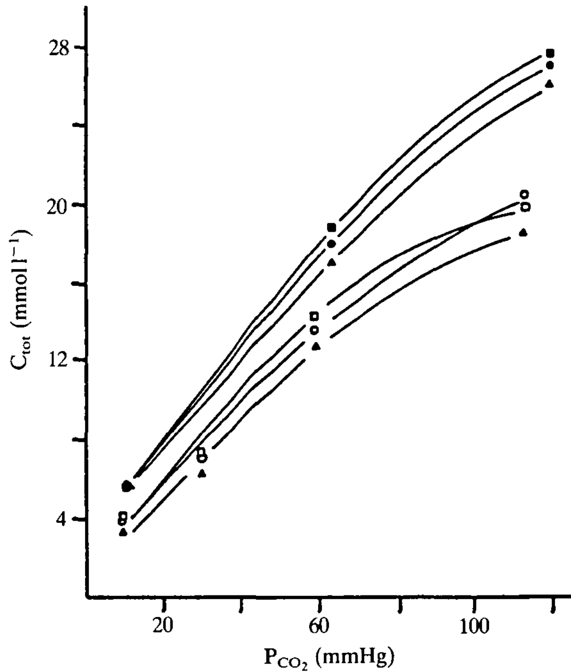

Fig. 2. CO_2 combining curves of *Melanoplus bivittatus* at 10°C (closed symbols) and 35°C (open symbols). Symbol type represents an individual pooled haemolymph sample.

Table 2. *The effect of temperature and fractional CO_2 in the equilibration gas on pH and $[HCO_3^-]$* in vitro

	10 to 25°C shift			25 to 35°C shift		
	ΔpH (units °C^{-1})	Δ$[HCO_3^-]$ (mmol l^{-1} °C^{-1})	$\frac{[HCO_3^-{}_{(25)}]}{[HCO_3^-{}_{(10)}]}$	ΔpH (units °C^{-1})	Δ$[HCO_3^-]$ (mmol l^{-1} °C^{-1})	$\frac{[HCO_3^-{}_{(35)}]}{[HCO_3^-{}_{(25)}]}$
2% CO_2	−0·0037 ±0·00112	−0·109 ±0·0294	0·67 ±0·106	−0·0039 ±0·00249	−0·017 ±0·0551	0·98 ±0·155
10% CO_2	−0·0022 ±0·0005	−0·220 ±0·0131	0·76 ±0·070	+0·0011 ±0·00546	+0·070 ±0·0200	1·09 ±0·035
18·7% CO_2	−0·0010 ±0·00095	−0·409 ±0·0337	0·68 ±0·038	+0·0070 ±0·003564	+0·20 ±0·010	1·14 ±0·029

Values are means ± S.D.
$N = 4$.

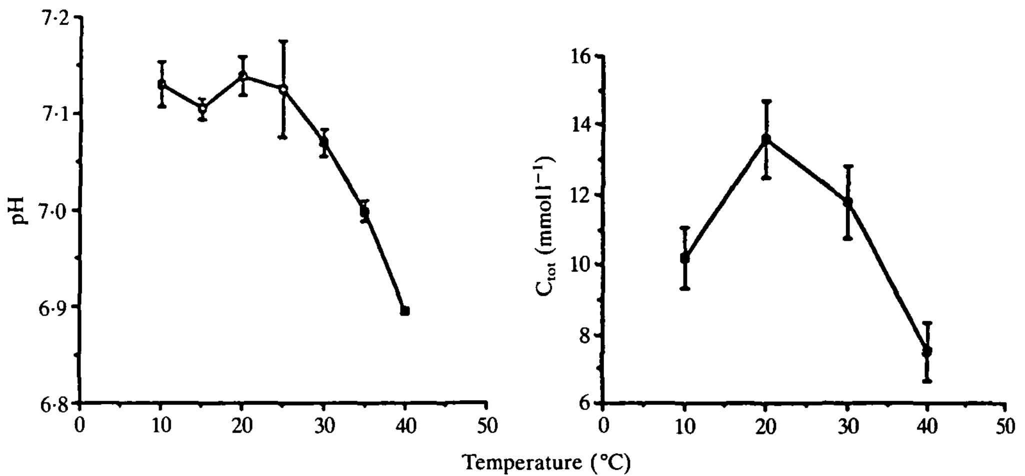

Fig. 3. Haemolymph pH and C_{tot} (mmol l^{-1}) of *Melanoplus bivittatus* as a function of body temperature (means and standard errors, $N = 7–10$ at each).

(ANOVA, $P < 0.001$). Haemolymph pH decreased significantly from 25 to 35°C, and from 35 to 40°C (Duncan's *a posteriori* test, $P < 0.05$). From 25 to 40°C, the slope of the pH change with temperature was −0·017 pH units°C^{-1}. Total CO_2 content increased significantly from 10·2 to 13·6 mmol l^{-1} between 10 and 20°C (Duncan's test $P < 0.05$), and then decreased to 7·5 mmol l^{-1} as temperature increased to 40°C (Duncan's test, $P < 0.05$; Fig. 3). Although true standard errors cannot be calculated for the P_{CO_2}, S (dissolved CO_2) and [HCO_3^-] data, as pH and C_{tot} were not measured in the same animal, estimated standard errors were calculated by using P_{CO_2} values calculated for randomly paired C_{tot} and pH values (Fig. 4). This procedure is supported by the lack of a significant correlation between pH and C_{tot} in individual locusts (Harrison, 1987).

Changes in the derived variables (P_{CO_2}, S, [HCO_3^-]) were analysed statistically using nonparametric rank tests (Mann–Whitney *U*). P_{CO_2} at 20°C was significantly higher than at 10°C ($P < 0.01$), but P_{CO_2} values at 20, 30 and 40°C did not differ significantly. Dissolved CO_2 declined significantly from 20 to 40°C ($P < 0.001$), but remained statistically constant from 10 to 20°C. [HCO_3^-] at 40°C was significantly lower than values at either 20 or 30°C ($P < 0.001$, $P < 0.05$, respectively).

Haemolymph protein content was unaffected by 12–16 h of temperature acclimation, and averaged 35·7 mg ml^{-1} (S.E. = 1·15; $N = 30$). Haemolymph total ammonia content increased from 139 μmol l^{-1} at 10°C (S.E. = 40·9; $N = 8$) to 500 μmol l^{-1} at 35°C (S.E. = 79·0; $N = 8$; Duncan's test, $P < 0.05$).

Discussion

Field body temperatures

The diurnal pattern of field body temperatures in *M. bivittatus* was similar to that

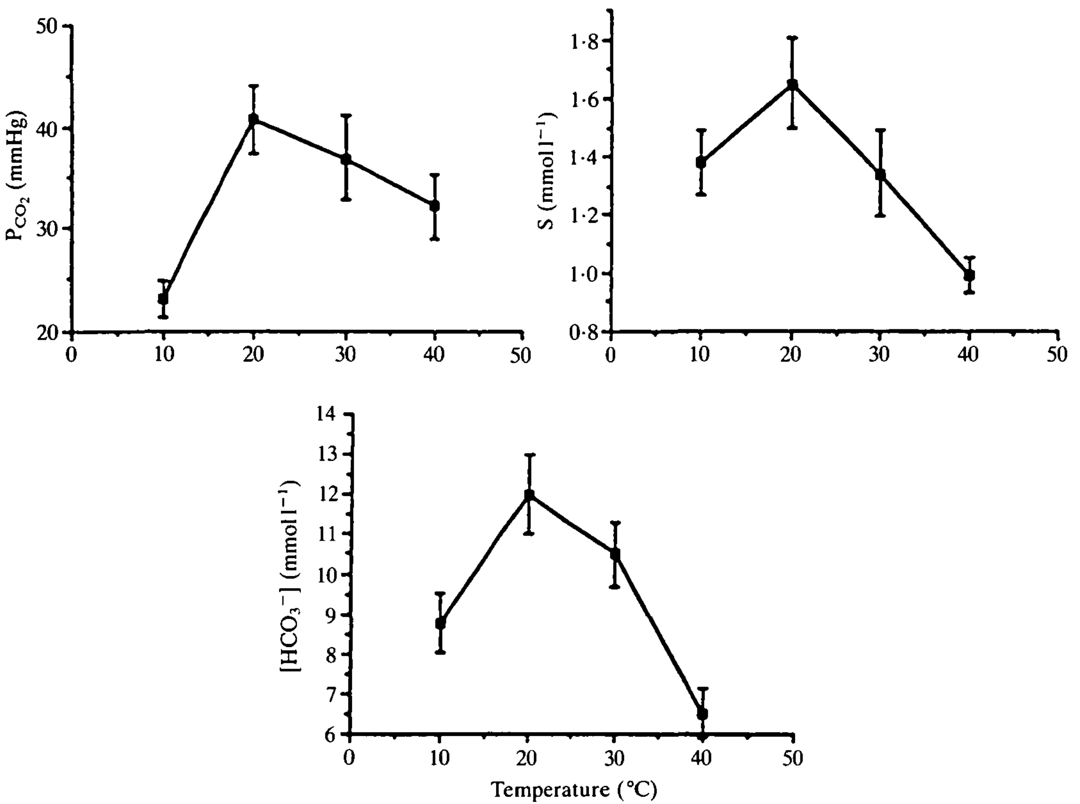

Fig. 4. P_{CO_2} (mmHg), S (dissolved CO_2, mmol l^{-1}) and [HCO_3^-] (mmol l^{-1}) calculated for *Melanoplus bivittatus* haemolymph at varying body temperatures (means and standard errors, $N = 7$–10 at each).

reported for other grasshoppers (reviewed by Uvarov, 1977). *M. bivittatus* experienced wide fluctuations in body temperature, indicating that these grasshoppers are particularly good models for testing the effect of temperature variation on acid–base physiology.

Haemolymph in vitro

The CO_2 solubility coefficients determined in acidified water were bracketed by previous values in the literature (Bartels & Wrbitzky, 1960; Murray & Riley, 1971, values revised by Weiss, 1974), indicating that the techniques utilized here provided satisfactory results despite the use of small samples. CO_2 solubility coefficients in *M. bivittatus* haemolymph were similar to those reported for vertebrate plasma (Bartels & Wrbitzky, 1960; Austin *et al.* 1963; Boutilier *et al.* 1985). Carbonic acid pK_{app} values in *M. bivittatus* haemolymph were also similar to values reported in the literature for vertebrates and crustaceans, and the dependence of pK_{app} on temperature ($-0{\cdot}00808$ pH units °C^{-1}) agrees well with that reported for vertebrate plasmas at constant pH (Severinghaus *et al.* 1956;

Albers & Pleschka, 1967; Boutilier *et al.* 1985). Apparent pK values similar to those of vertebrates have also been reported for *Gastrophilus* larvae (Levenbrook, 1950) and honeybee larvae (Bishop, 1923).

The haemolymph nonbicarbonate buffer value of *M. bivittatus* is among the highest reported for any extracellular fluid ($-35\,\mathrm{mequiv\,l^{-1}\,pH\,unit^{-1}}$). The specific buffer value per gram of haemolymph protein, $0{\cdot}76\,\mathrm{mequiv\,g^{-1}\,pH\,unit^{-1}}$, is exceedingly high when compared with the value obtained for human haemoglobin ($0{\cdot}13\,\mathrm{mequiv\,g^{-1}\,pH\,unit^{-1}}$; Adair, 1925). However, comparisons with vertebrates are difficult as invertebrate protein contents estimated from bovine serum albumin standards underestimate gravimetric measures by 25–90 % (Marsh, 1987).

In other species, for example in a crab and in the tuna, extracellular fluid pH decreases from $-0{\cdot}007$ to $-0{\cdot}013$ pH units $°C^{-1}$ *in vitro* at constant P_{CO_2} (Wood & Cameron, 1985; Perry *et al.* 1985). As P_{CO_2} *in vivo* in *M. bivittatus* is statistically constant above 20°C, it is important to know the effect of temperature on pH and [HCO_3^-] at constant P_{CO_2} to distinguish active pH regulation from such passive, physicochemical effects. The change in pH with temperature *in vitro* at constant P_{CO_2} did not exceed $-0{\cdot}004$ pH units $°C^{-1}$, indicating that the excess changes in pH with temperature *in vivo* must be due to active processes.

Haemolymph in vivo

Critique of methods

Although the haemolymph sampling procedure was clearly not anaerobic, preliminary studies indicated that, if the haemolymph droplet was sampled quickly, C_{tot} loss was minimal. This is apparently due to the lack of carbonic anhydrase in insect haemolymph (Levenbrook & Clark, 1950). Haemolymph droplets of smaller size than those sampled from the neck (20 μl) were equilibrated to a P_{CO_2} near the maximum observed *in vivo* (47 mmHg), and then exposed to air for varying times before sampling for C_{tot}. If the droplet was sampled in less than 10 s, the loss of C_{tot} was less than 3 % (J. M. Harrison, unpublished data).

A second important consideration is whether this sampling procedure allows collection of 'resting' haemolymph. In vertebrates, it has been well documented that catheterization is necessary to obtain blood at resting acid–base status (reviewed by Heisler, 1984). This might be expected to be a particular problem with insects, which are known for their capacity for rapid changes in metabolic rate. Grasshopper activity is accompanied by CO_2 accumulation which accounts quantitatively for haemolymph acidosis during activity (Harrison, 1987). In preliminary experiments, I found that elevations in haemolymph C_{tot} (and depression of pH) were found only after 20 s of handling. Haemolymph samples obtained from grasshoppers handled for 2–15 s were identical in acid–base status (J. M. Harrison, unpublished data). Therefore, it is reasonable to argue that the haemolymph samples obtained from animals with minimal handling time (<10 s) approximated 'resting' haemolymph in terms of acid–base status. The absolute

values measured in this study for haemolymph pH and [HCO_3^-] at 20°C (7·121, 11·9 mmol l^{-1}, respectively) agree well with those reported for the locust, *Schistocerca gregaria* (7·1, 13 mmol l^{-1}, respectively; Phillips *et al.* 1986). The low absolute value of haemolymph pH appears to be a general characteristic of insect haemolymph (Buck, 1953).

The effect of temperature of haemolymph acid–base status in vivo

Between 25 and 40°C, the change in pH with temperature conserves the calculated fractional dissociation state of imidazole quite well, as it varies by only 1·4 % around its mean of 0·606 between 25 and 40°C. This calculation must be viewed with some caution, however, given the diversity of values for the temperature-dependence of pK among imidazoles (Edsall & Wyman, 1958). Relative alkalinity is also well conserved, varying approximately 6 % around the mean value over this temperature range. Preservation of fractional protein dissociation has been shown to preserve the functional and structural properties of imidazole-containing proteins, and to be theoretically important in the preservation of the capacity for control of metabolic flux (Wilson, 1977*a*,*b*; White & Somero, 1982). However, between 10 and 25°C, pH is statistically constant and both relative alkalinity and the calculated fractional dissociation of imidazole groups change significantly, with a decrease in calculated fractional dissociation of imidazole groups of 27 % at 10°C. Relative alkalinity at 10°C is reduced to 25 % of its value at 40°C.

In most grasshoppers, feeding and locomotory movements only occur above approximately 20°C (Uvarov, 1977), and this is also true for *M. bivittatus*. The data suggest that *M. bivittatus* only regulate haemolymph pH in a manner which preserves relative alkalinity and the fractional dissociation of proteins at temperatures corresponding to daily activity temperatures. This result is similar to that reported for the lizard, *Dipsosaurus dorsalis* (Bickler, 1981, 1982).

Mechanisms of haemolymph pH regulation with temperature

The mechanism by which *M. bivittatus* changes haemolymph acid–base status with temperature is quite different from the 'constant CO_2' system described for most air-breathers (Reeves, 1977). P_{CO_2} changes with temperature are inconsistent with those required to accomplish the observed pH changes with temperature *in vivo*. The decrease in pH between 20 and 40°C is accompanied by a statistically constant P_{CO_2}, whereas the constant pH between 10 and 20°C is accompanied by increasing P_{CO_2}. Thus the changes in haemolymph pH with temperature cannot be explained by variation of ventilation relative to metabolic CO_2 production.

Although the change in pH with temperature at constant P_{CO_2} *in vitro* was not measured at the exact P_{CO_2} values occurring *in vivo*, it is reasonable and conservative to assume that the passive, physicochemical $\Delta pH\,°C^{-1}$ will be between the maximum and minimum measured (+0·0010 and −0·0039 pH

unit °C^{-1}, Table 2). [HCO_3^-] may than be predicted from the change in CO_2 solubility coefficients and carbonic acid pK values:

$$[HCO_3^-{}_{(t2)}] = [HCO_3^-{}_{(t1)}]\alpha_2/\alpha_1 10^{(\Delta pK + \Delta pH)} .$$

This equation gave a good fit between observed and expected [HCO_3^-] after temperature changes *in vitro* ($r^2 = 0{\cdot}97$). Net bicarbonate additions or removals were then calculated using standard methods (Davenport, 1974). The transition from 10 to 20°C *in vivo* is accompanied by an active net addition of 4·2–6·2 mmol l^{-1} of bicarbonate equivalents to the haemolymph. The ionic alkalinization is compensated with an increase in P_{CO_2}, resulting in constant pH. From 20 to 30°C there is a net removal of 1·1–3·6 mmol l^{-1} of bicarbonate equivalents from the haemolymph, and from 30 to 40°C there is a further net removal of 8·8–11·2 mmol l^{-1} of bicarbonate equivalents. Cumulative errors of 1 standard deviation for the *in vivo* acid–base measurements would increase the range of these estimates by approximately 2 mmol l^{-1}.

These changes in amount of extracellular bicarbonate could be due to changes in metabolic production and utilization of acid–base equivalents within the haemolymph. However, this seems unlikely given the low cell fraction of grasshopper haemolymph (<3 %). A more likely explanation is that the changes in haemolymph bicarbonate are due to transmembrane transfer of acid–base-relevant ions, either between the intra- and extracellular compartments and/or between the haemolymph and the lumen of the digestive tract. Locust recta possess a large capacity for H^+ excretion, which may be due to an electrogenic H^+-ATPase and a Cl^-/HCO_3^- antiport (Phillips *et al.* 1986). The faeces of locusts are generally acidic, suggesting that a constant excretion of protons may be necessary for pH homeostasis in these animals (Phillips *et al.* 1986). Further studies of haemolymph ion activities, the temperature-dependence of net proton release by the intracellular space and net proton elimination by the excretory system will be necessary to determine the exact mechanism of haemolymph pH regulation with temperature in *M. bivittatus*.

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