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GEOGRAPHIC AND ALTITUDINAL VARIATION IN WATER BALANCE AND METABOLIC RATE IN A CALIFORNIA GRASSHOPPER, *MELANOPLUS SANGUINIPES*

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Summary

The importance of respiratory patterns and the physical properties of cuticular lipids to insect water balance were investigated in natural populations of the grasshopper *Melanoplus sanguinipes*. I specifically test the hypotheses that patterns of discontinuous ventilation affect water loss and that increased amounts and melting points of cuticular lipid reduce water loss. Using flow-through respirometry, rates of water loss and carbon dioxide release from grasshoppers were quantified at 25, 35 and 42 °C. Populations displayed substantial variation, with high-elevation populations exhibiting the greatest water loss and metabolic rates. Behavior leading to discontinuous gas exchange was observed in several populations, but its occurrence decreased dramatically at high temperatures and was not correlated with a reduction in the rate of water loss. The amount and melting point of cuticular lipids were determined for each individual using gas chromatography and Fourier-transform infrared spectroscopy. Increased amounts and higher melting points of cuticular lipids were strongly correlated with lower rates of water loss in populations. I show that discontinuous gas exchange is unlikely to be a mechanism for reducing water loss in these insects and that the lipid properties are primarily responsible for variation in overall water loss rates.

Key words: *Melanoplus sanguinipes*, discontinuous ventilation, discontinuous gas-exchange cycle, elevation, water loss, cuticular lipid, metabolic rate, grasshopper.

Introduction

Two of the oldest and most central hypotheses in insect physiology arose to explain the ability of many species to flourish in hot, arid environments, despite strong physiological challenges to maintaining water balance in such small organisms. These involve the role of the cuticle as a barrier to water loss and the role of respiratory patterns in reducing water efflux.

Relative to their small body size, insects expose large surfaces to potential evaporative water loss, both from respiratory structures and across the integument itself. The development of a wax-covered, impermeable cuticle highly resistant to water loss is cited as one of the most crucial and far-ranging adaptations for all arthropods (Hadley, 1985, 1994a; Chapman, 1998), enabling a terrestrial lifestyle and allowing greater radiation into xeric environments. Even in more mesic species, the barrier to evaporation provided by the epicuticular hydrocarbon layer is thought to be an important mechanism for reducing overall water loss.

The second proposed adaptation for resistance to desiccation is a behavioral modification of the ventilatory pattern whereby the loss of water from the tracheal system is minimized through periodic closure of the spiracular openings, causing short-term hypoxia and hypercapnia. This results in large bursts of cyclical carbon dioxide release and oxygen uptake, punctuated by periods of little or no gas exchange. This pattern is termed discontinuous ventilation or, more recently (Lighton 1994; Lighton and Garrigan, 1995), the discontinuous ventilatory cycle (DVC) or discontinuous gas-exchange cycle (DGC). Purported water savings occur during the phase when the spiracles are closed and respiratory transpiration is essentially zero, and also during a ‘flutter’ phase during which oxygen is believed to be taken up with little water loss.

Comments on the importance of the cuticular wax layer began in earnest with observations by Ramsay (1935), Wigglesworth (1945) and Lees (1947) on changes in the permeability of the cuticle following abrasion or exposure to increasing temperature. The phenomenon of a critical temperature for a transition in water loss rate (WLR) was noted by Wigglesworth (1945) and others (Loveridge, 1968; Hadley and Quinlan, 1989). Water loss rates measured by a variety of means from disparate groups of arthropods remained low as temperature was elevated and then abruptly increased as a critical temperature was reached (for reviews, see Hadley, 1994a; Rourke and Gibbs, 1999).

A three-dimensional molecular packing model has been proposed to explain the transition phenomenon. Central to the

theory is the manner in which the lipid components are organized in the layer and the resultant size of the inter-molecular gaps presented for water molecules to pass. As the lipid layer proceeds through a phase transition from crystalline gel to fluid, such as occurs with increasing temperature near the melting point of a mixture, the molecular gaps would become larger and permeability to water would increase. Certain types of hydrocarbon compounds, such as non-branched alkanes, possess both higher melting points and closer packing characteristics than methyl-branched or unsaturated hydrocarbons (Gibbs and Pomonis, 1995). The physical properties of the lipids, specifically melting point as it is influenced by hydrocarbon composition, are directly responsible for the degree of cuticular permeability. Strong support for the effect of temperature on the phase transition in the molecular packing model has been presented recently using simple membrane and *in situ* techniques (Rourke and Gibbs, 1999). However, the importance of this model and the specific effects of the cuticular lipid melting point on water loss have not been adequately tested in natural populations of insects.

Greater amounts of lipid and a higher lipid melting point (T_m) should serve to reduce cuticular water loss rates (Lockey, 1988; Noble-Nesbitt, 1991; Hadley, 1994a). Many studies have characterized differences in lipid composition among insect species (Hadley, 1981; Lockey 1985, 1988; Blomquist et al., 1987; de Renobales et al., 1991), and some have correlated lipid composition with cuticular permeability (Hadley, 1978; Toolson and Hadley, 1979; Toolson, 1984). Water loss differences can be attributed to lipid properties in *Drosophila pseudoobscura* (Toolson, 1982; Toolson and Kuper-Simbrón, 1989), but other studies in both wild and laboratory-selected *Drosophila* have not been able to establish correlations (Gibbs et al., 1997, 1998). A complete explanation of the importance of cuticular properties to insect water loss has not yet been convincingly presented.

The cyclic release of carbon dioxide was observed and characterized decades ago in lepidopteran pupae (Buck, 1962; Levy and Schneidermann, 1966) and in grasshoppers (Hamilton, 1964). The occurrence of the DGC as a ventilatory pattern is widespread among insects (Miller, 1981) and was hypothesized to be an adaptive mechanism for reducing water loss rates (for reviews, see Hadley, 1994b; Lighton, 1994; Lighton and Berrigan, 1995). But while Lighton et al. (1993a) found strong support for the role of the DGC in water saving, many more studies have failed to find any correlation (Williams and Bradley, 1998; Williams et al., 1998; Lighton, 1998) or have even contradicted the theory (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993; Lighton et al., 1993b; Lighton and Garrigan, 1995). This has brought about a revision of the assessment of the adaptive role of the DGC such that it is thought to be important only for individuals when respiratory water loss is a large percentage of total water loss or, similarly, when transcuticular permeability is low.

The grasshopper *Melanoplus sanguinipes* (Orthoptera, Acrididae) is a particularly useful and appropriate study organism. The species is abundant and widely distributed throughout North America (Vickery and Kevan, 1983). Individuals can be found in a variety of differentiated habitats and display marked variation in cuticular lipid composition and T_m (Gibbs et al., 1991; Gibbs and Mousseau, 1994). In the present study, I have chosen to exploit this inter-individual variation to measure T_m, the amount of lipid and respiratory variables from the same individual. My goal was to determine the relative functional significance of each variable in the water economy of this insect and to determine whether the relative significance varies among individuals in a population.

In this study, I collected grasshoppers from several warm, coastal and inland sites and from two high-elevation alpine sites. I investigated potential differences in water loss rate and the physiological effects of ventilation pattern and cuticular lipid properties. Specifically, I directly tested the hypothesis that lipid extracts with higher T_m confer lower permeability and WLR, and correlate WLR with environmental variables. I also tested the hypothesis that the discontinuous ventilation cycle is a plausible mechanism for reducing respiratory WLR. I report on differences in metabolic rate estimates among grasshoppers from lowland and montane habitats, and present a synthesis of the factors affecting WLRs in natural populations of insects.

Materials and methods

Grasshopper collection and husbandry

Adult grasshoppers *Melanoplus sanguinipes* from California, USA, were caught by hand between the months of June and August in 1996, 1997 and 1998. The six population field sites are listed in Table 1, with geographic location and elevation. The Santa Rosa Ecological Plateau (SR) is a joint Nature Conservancy and University of California Natural Reserve System site. Crooked Creek (CC) is part of the White Mountain Research Station of the University of San Diego. The other sites are all part of the University of California Natural Reserve System: Angelo Coast (AC), Hastings (HA), Motte Rimrock (MR) and Sierra Nevada Aquatic Research Laboratory (SN). Two of the sites, CC and SN, can be characterized as having cooler average temperatures and high elevations. The other sites are relatively warmer, low-altitude

Table 1. *Locations of collecting sites for grasshoppers*

Location	Latitude	Longitude	Elevation range (m)
Angelo Coast	39°43′45″N	121°38′40″W	378–1290[a]
Hastings	36°12′30″N	121°33′30″W	144–293
Motte Rimrock	33°48′45″N	117°15′30″W	482–605
Santa Rosa	33°48′45″N	117°15′30″W	370
SN	37°36′51″N	118°49′47″W	2177
Crooked Creek	37°29′57″N	118°10′14″W	3123

[a]Approximate elevation of collecting site was 500 m.
SN, Sierra Nevada Aquatic Research Laboratory.

locations. These observations are supported by climate data provided by the reserve managers, in some cases dating back five decades.

Grasshoppers were transported to Irvine, California, in plastic cages or, in some instances, were air-mailed by overnight delivery. The insects were maintained for up to 2 weeks in individual plastic cages at 25 °C on a 14 h:10 h L:D photoperiod and fed a diet of fresh romaine lettuce *ad libitum*.

Grasshopper field body temperature

On several occasions, grasshopper body temperatures were sampled at the collection sites, using a small, needle-thermocouple insect probe (Physitemp, USA). Between approximately 09:00 h and 17:00 h on a single day, adult grasshoppers were caught by hand in a small net and quickly stabbed in the thorax with the probe. The needle was inserted approximately 2 mm into the body and held there briefly. This was completed during the first 5–10 s of capture, before the body temperature could change significantly in response to handling. The temperature was noted on a digital thermometer, together with air temperature (at 1 cm and 1 m above soil) and soil temperature at the particular spot where the insect was first netted.

To ascertain the range of available temperatures that grasshoppers could choose through behavioral thermoregulation, five miniature temperature loggers (Onset Data Corporation, Massachusetts, USA) were each placed on the ground, in full sun and in the shade, at locations around the fields where the grasshoppers were collected. Temperatures were sampled with a thermistor probe every minute, and the data were stored in the logger memory chip until retrieval. The average of all sun data and all shade data from the loggers on a particular day was then plotted with grasshopper body temperatures (T_b).

Several adult grasshoppers were killed with cyanide vapor and placed in a convection oven to dry the carcass. These were fashioned into thermal mannequins by attaching a small-gauge thermocouple to the thorax, which was inserted through a small hole made with a pin and sealed with silicone. Although the coloration of the cuticle darkened somewhat after drying, the legs and wings remained intact and could be posed in a resting posture. Potential evaporative cooling effects are minimal (A. G. Gibbs, personal communication), and Prange (1996) has estimated that insects of this relatively small size are probably unable to utilize evaporative cooling.

Average 24 h shade air and soil temperature data were also collected by placing additional temperature loggers, which were left on site for a month or more before retrieval. These data were used to supplement the historical climate data and, in cases where historical and data-logger temperatures were both available, they closely matched the data collected from permanent weather stations at the reserve sites.

Determination of grasshopper water loss and carbon dioxide release

Flow-through respirometry was used to measure whole-insect rates of water loss and carbon dioxide release at 25, 35 and 42 °C. Grasshoppers collected during 1996 were used for respirometry runs at 25 °C in a temperature-controlled room. Individuals collected during 1997 and 1998 were used for runs in a Peltier-effect temperature-controlled cabinet (Sable Systems, Nevada, USA) at 35 and 42 °C, respectively. The temperature cabinet was itself housed in the temperature-controlled room, together with the electronics used for data collection and control of the cabinet.

Room air was pumped by an air compressor through two 1 l canisters of silica gel drying agent at 100 ml min^{-1}; the airflow was then split into one sample and one flushing line. The sample airflow passed through another canister filled with a carbon dioxide scrubber, Ascarite and Drierite drying agent. Sample air was directed to an infrared carbon dioxide and water analyzer (Licor Instruments, model LI-6262). The analyzer was connected to a PC running data-acquisition software (Sable Systems), and data were sampled every 2 s. Calibration of the water sensor was performed at the beginning of each group of respirometry temperatures and was carried out by injecting known volumes (0.5–3.0 μl) of water into the air line. Calibration of the carbon dioxide sensor was performed using gas mixtures of known concentration.

Grasshoppers were fasted for 24 h before being placed into individual 5 ml glass respirometry chambers. The chambers were connected by short sections of low-hygroscopic rubber tubing to a solenoid-activated multiplexer. The multiplexer unit allowed the sample airflow to be switched from chamber to chamber and was automated by software running on a PC. Chambers that were not being sampled were continuously flushed by the dry air from the pump, also at 100 ml min^{-1}. During the runs at 35 and 42 °C, the entire multiplexer assembly was housed within the temperature-controlled cabinet. Eight chambers could be sampled during a run, but typically five or six chambers held insects and the other empty chambers were used as blanks to establish baselines for water loss and carbon dioxide release. Each chamber was sampled for 30 min before the sample airflow switched to the next chamber; each insect was therefore recorded every 4 h.

Respirometry runs lasted for 20 h, with data analyzed only for the period from 12:00 to 20:00 h. This allowed the insects to become accustomed to the chambers and coincided with what would normally be a period of low activity (22:00 h to 06:00 h). Runs were conducted in the dark, which also seemed to reduce activity in the chambers. The rates of water loss from early portions of the run were relatively high compared with later measurements, possibly reflecting a release of water from the cuticle itself. It has been suggested that this does not represent respiratory or transcuticular water loss (Hadley, 1994a), but rather the release of water from the slightly hygroscopic cuticle, which had previously absorbed atmospheric water vapor.

For each insect, the average of two 30 min recordings was taken. Portions of the recording in which the insect was not stationary could be identified and were removed from the average. Defecations, regurgitation of fluid and egg-laying

could also be identified and were removed from the water loss analyses. Signal noise was very low for CO_2, averaging less than 0.01 µl h^{-1}; the water loss signal noise was comparable. Because the water loss sample signal was much smaller than the CO_2 signal, the error for WLR was larger, approximately 1–5 %.

Determination of cuticular lipid properties

Grasshoppers were placed in a freezer at −70 °C immediately after the respirometry runs. They were later thawed and immersed for 15 min in a hexane wash at room temperature (23–25 °C) to extract cuticular lipids. The hexane was evaporated under nitrogen gas, and lipid extracts were themselves stored at −70 °C until analysis. Samples were dissolved again in hexane solution at room temperature and split into two equal volumes before analysis. A hydrocarbon standard of known length (*n*-docosane, C_{22}, Aldrich Chemicals) was added to one volume, delivered as either 25 or 50 µg in a small drop (0.5 µl) of hexane, using a micropipette.

Cuticular lipid melting temperature, T_m, was determined using Fourier-transform infrared spectroscopy (Gibbs and Crowe, 1991). The total amount of lipid was quantified by gas chromatography (Hewlett-Packard model 5890A, 30 m×0.32 µm capillary column) using the lipid samples with the hydrocarbon standard.

Results

Male *versus* female analyses of covariance for T_m, the amount of lipid, WLR and metabolic rate did not show any differences within a population. The average mass of individuals was significantly greater in the two high-elevation populations, CC and SN (analysis of variance, ANOVA, $P<0.005$) during 1997.

Grasshopper field body temperature

Body temperature measurements using the above protocol were performed for 2 days at each of the Sierra Nevada (SN) and Santa Rosa (SR) field sites during August 1998; representative data from each location are shown in Fig. 1. Additional T_b measurements were performed sporadically during 1996–1998 at all locations except for Crooked Creek (CC). In all sites sampled, grasshopper T_b values above 40 °C could regularly be found; the maximum T_b values recorded were 44.6 °C at SN and 46.4 °C at SR. In nearly every measurement taken, the insect body temperature was higher than the ambient air temperature at the location of capture and, typically, slightly cooler than the soil temperature. Mean T_b at SN over two consecutive days was 40.1±0.56 °C (N=33), and mean T_b at SR for two days was 41.1±0.36 °C (means ± S.E.M., N=51). Results using a grasshopper thermal model showed that maximum T_b could exceed 50 °C in full sun at both sites.

Whole-insect water loss rates

Water loss was measured from three populations at 25 °C (MR, SN and SR), from five populations at 35 °C (AC, CC, HA, SN and SR), and from two populations at 42 °C (SN and SR). Water loss rates (WLRs) measured from all populations at 25 °C (N=114) and 35 °C (N=116) are plotted in Fig. 2. Multiple pairwise comparisons, using Bonferonni corrections, were made between populations, using mass as a covariate. The statistics software package Systat 8.0 (SPSS) was used, and outliers identified by the software were removed from

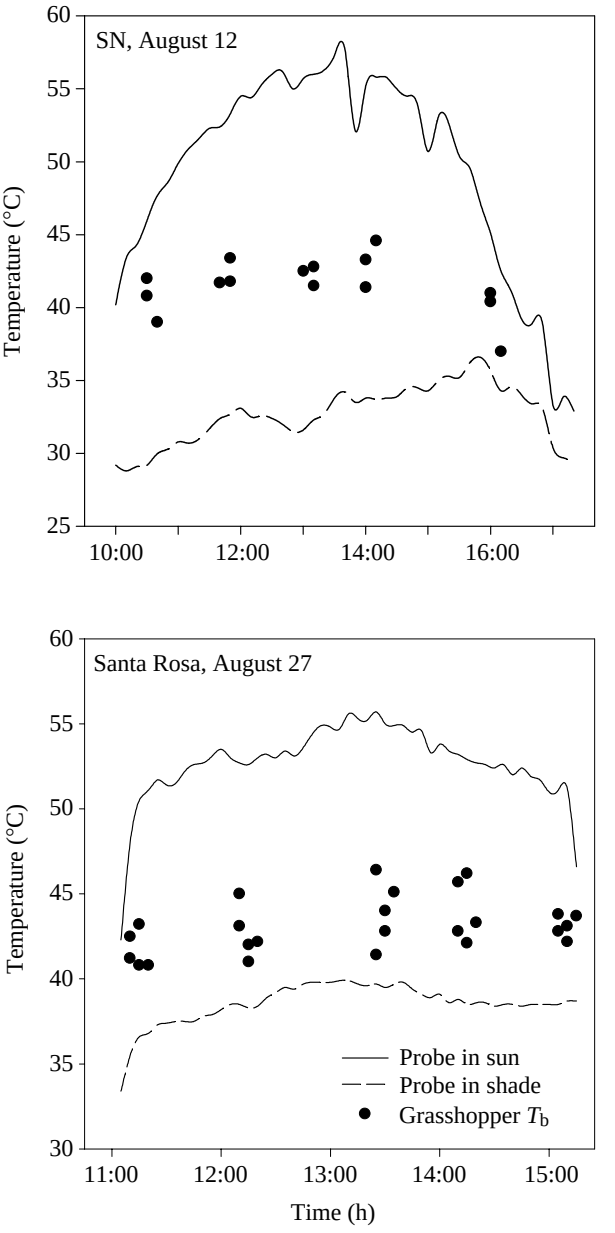

Fig. 1. Representative data from typical days of grab-and-stab body temperature measurements. Grasshopper body temperature (T_b) (filled circles) is shown for a high-elevation population (Sierra Nevada Aquatic Research Laboratory, SN) (A) and a low-elevation population (Santa Rosa) (B). Probe temperature measured 1 cm from the ground every minute is shown for full sun (solid lines) and shade (dashed lines). Mean T_b values during this period were 40.1±0.56 °C (N=33) for SN and 41.1±0.36 °C (means ± S.E.M., N=51) for Santa Rosa.

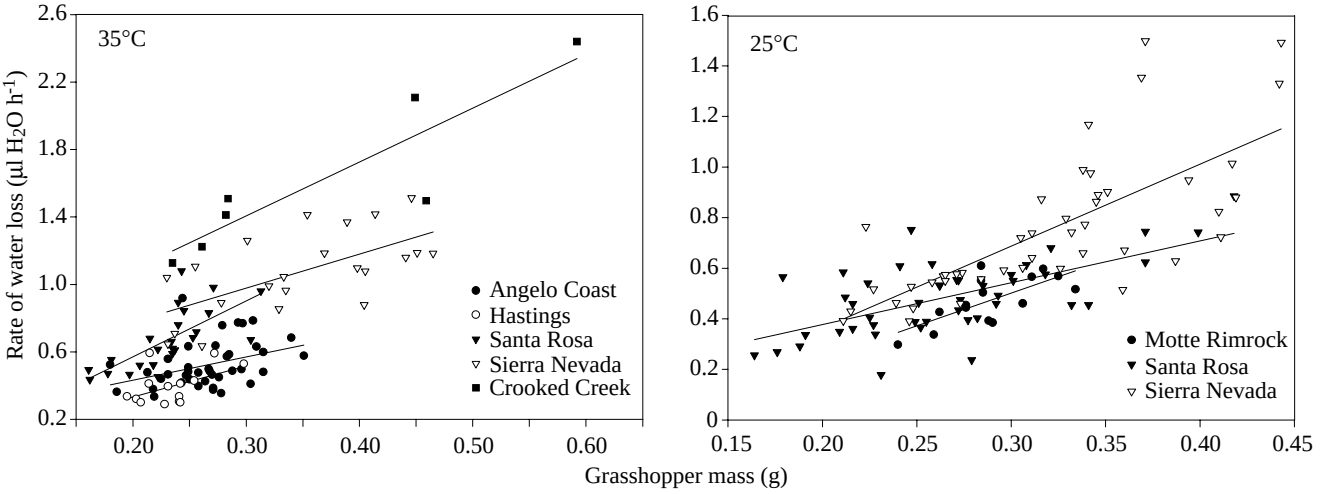

Fig. 2. Individual water loss rates from several populations at two different respirometry temperatures. Data at 25 °C (N=114) were collected during 1996, and data at 35 °C (N=116) were collected during 1997. Water loss rate for each individual represents an average of two 30 min recordings, taken after 12 and 16 h in a flow-through respirometry chamber at an airflow of 100 ml min^{-1}. Significant differences are evident among populations (ANCOVA, P<0.0001), with the higher-elevation populations, Sierra Nevada and Crooked Creek, consistently displaying the highest rates of water loss. All other populations are from lower-elevation, warmer field sites. For regression data, see Table 2.

consideration. The analyses of covariance (ANCOVA) revealed significant differences between the populations at both temperatures (P<0.0001) except for three instances: MR *versus* SR at 25 °C (P=0.054), and AC *versus* HA (P=0.068) and SN *versus* SR (P=0.052) at 35 °C. There were no apparent differences in WLR at the highest temperature, 42 °C (N=43). The high-altitude populations, SN and CC, consistently showed the highest WLRs of any individuals. Regressions of mass against WLR were all highly significant (P<0.01)

For two populations, SN and SR, WLRs were compared for all three temperatures (Fig. 3). Pairwise comparisons with mass as the covariate show that WLRs were significantly different among temperatures (ANCOVA, P<0.0001) for both populations. Water loss increased in a similar fashion for each population, on average by 46 % between 25 and 35 °C and by 480 % between 25 and 42 °C. Regression data and statistics for water loss rates and carbon dioxide release are summarized in Table 2.

The repeatability of WLR measurements was tested by performing three respirometry runs at 35 °C on a small group of grasshoppers (N=15) at approximately 2 week intervals. No differences were detected between the runs, and I believe the

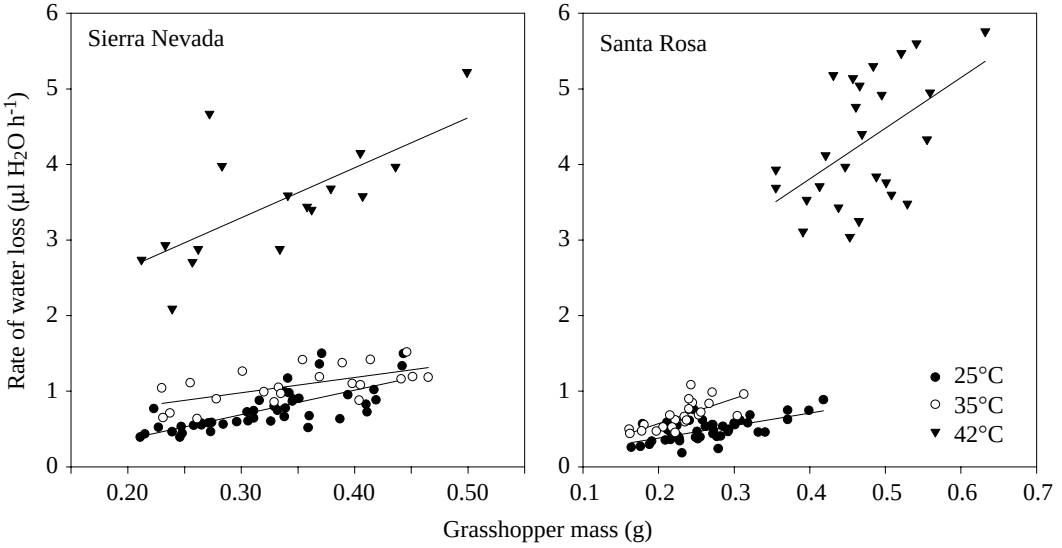

Fig. 3. A comparison of rates of water loss at three temperatures from two populations (N=178). The Sierra Nevada population is at higher elevation than the Santa Rosa population and experiences a very different climate. Populations show significant differences in water loss only at 35 °C (ANCOVA, P<0.0001). The large increase in rates of water loss for both populations at 42 °C can be attributed to a partial melting of the cuticular lipid layer, resulting in an increase in permeability. Regression data are given in Table 2.

Table 2. *Regression analyses of rates of water loss and carbon dioxide release against mass*

Location	Temperature (°C)	WLR equation	*P* value	CO_2 equation	*P* value
Angelo Coast	35	y=0.16+1.38x	0.017	y=0.001+0.28x	<0.001
Hastings	35	y=–0.04+1.85x	0.078	y=0.01+0.26x	<0.001
Motte Rimrock	25	y=–0.27+2.58x	0.003	y=0.01+0.18x	0.15
Santa Rosa	25	y=0.05+1.65x	<0.001	y=0.00+0.23x	<0.001
	35	y=–0.09+3.32x	<0.001	y=0.01+0.29x	<0.001
	42	y=1.12+6.71x	0.007	y=0.05+0.49x	<0.001
SN	25	y=–0.28+3.23x	<0.001	y=0.35+0.19x	<0.001
	35	y=0.37+2.01x	0.001	y=0.06+0.18x	<0.001
	42	y=1.31+6.62x	0.003	y=0.11+0.45x	0.001
Crooked Creek	35	y=0.45+3.2x	0.006	y=0.1+0.15x	0.023

WLR, water loss rate.
SN, Sierra Nevada Aquatic Research Laboratory.

respirometry system to be a robust and sensitive technique. To verify that WLRs did not change significantly within adults of a natural population during a particular year, three separate field collections were taken from SN and two from SR in 1996 for respirometry at 25 °C. No differences could be detected between WLRs measured 2 months apart in the same populations.

As carbon dioxide production was recorded simultaneously with water loss, it was possible to differentiate between respiratory water loss and cuticular water loss. The average WLR when carbon dioxide release fell to zero was compared with the total WLR in the same individual, but only if the grasshopper demonstrated discontinuous gas exchange. At 25 °C, cuticular water loss averaged 85 % of total water loss, and varied among individuals within the range 71–96 % (N=50). At 35 °C, cuticular water loss averaged 79 % of total WLR, with individuals showing a range of 53–98 % (N=30). There were no differences apparent between the two populations, and no analysis was permitted at 42 °C since carbon dioxide release never fell to zero in any individual grasshopper.

Patterns of respiration and indirect metabolic rate estimates

Carbon dioxide release was used to examine respiratory patterns and, indirectly, to estimate metabolic rate. Estimates of metabolic rates plotted as CO_2 release for all populations are shown in Fig. 4. Populations were compared in pairwise fashion at each respirometry temperature, as above, with mass as the covariate. Analyses of covariance show significant differences in metabolic rates between populations (ANCOVA, P<0.0001), with four exceptions: MR *versus* SR

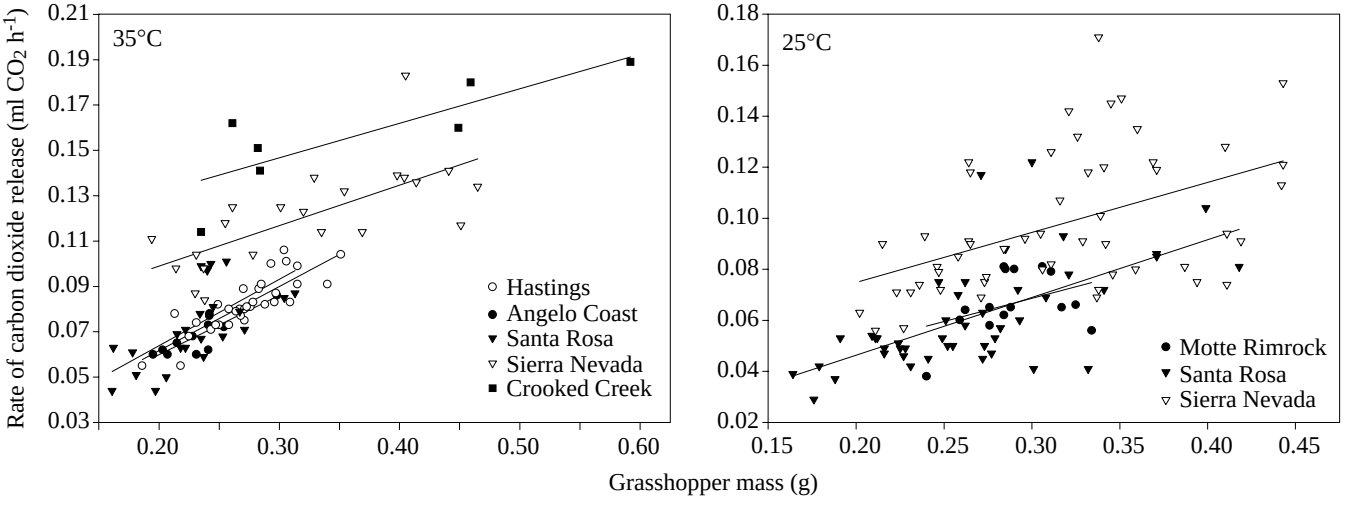

Fig. 4. Individual metabolic rates measured indirectly as rates of carbon dioxide release, from several populations at two different respirometry temperatures. Data at 25 °C (N=114) were collected during 1996, and data at 35 °C (N=116) were collected during 1997. The metabolic rate for each individual represents an average of two 30 min recordings, taken after 12 and 16 h in a flow-through respirometry chamber at 100 ml min^{-1}. Significant differences are evident among populations (ANCOVA, P<0.0001), with the higher-elevation populations, Sierra Nevada and Crooked Creek, showing the highest absolute and mass-specific metabolic rates. Regression data for each population are given in Table 2.

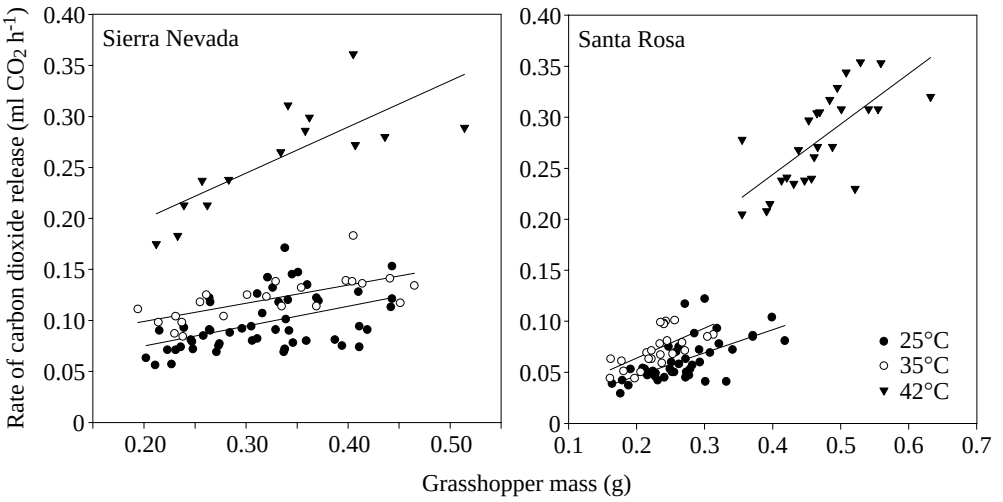

Fig. 5. Comparison of metabolic rates from three different respirometry temperatures, showing significant differences between populations (ANCOVA, P<0.001). Data for each temperature are from consecutive years, 1996–1998 (N=193). The high-elevation Sierra Nevada population shows consistently higher metabolic rates.

(P=0.386) at 25 °C, and AC *versus* HA (P=0.268), AC *versus* SR (P=0.638) and HA *versus* SR (P=0.462) at 35 °C. Regressions of metabolic rate against mass were highly significant (P<0.001). Only two regressions of mass-specific WLR and mass-specific metabolic rate were significant, AC at 35 °C (P=0.013) and SR at 25 °C (P=0.008). At 35 °C, a strong positive correlation was also found between metabolic rate and elevation (y=5.108x+0.286, r^2=0.96, P=0.003).

The metabolic rates of two populations, SN and SR, were compared at all three temperatures (Fig. 5); the high-altitude population consistently showed higher rates. For both populations, metabolic rates differed significantly at each temperature (ANCOVA, P<0.0001) The thermal coefficient, Q_{10}, was similar between populations, but varied over the temperature range investigated: 25–35 °C (SR=1.37, SN=1.2), 35–42 °C (SR=2.47, SN=3.07) and 25–42 °C (SR=1.74, SN=1.77). Mean Q_{10} values were 1.86 for SR and 2.01 for SN (N=3).

The relative occurrence of cyclic CO_2 release differed both among populations and between populations at different temperatures (Fig. 6). Grasshoppers from all populations except for Crooked Creek displayed bouts of discontinuous gas exchange, during which the rate of CO_2 release fell to zero for as long as 6–8 min. More commonly, the rate of CO_2 release remained at zero for only 2–3 min. The behavior was prevalent at 25 °C, persisted somewhat at 35 °C and disappeared

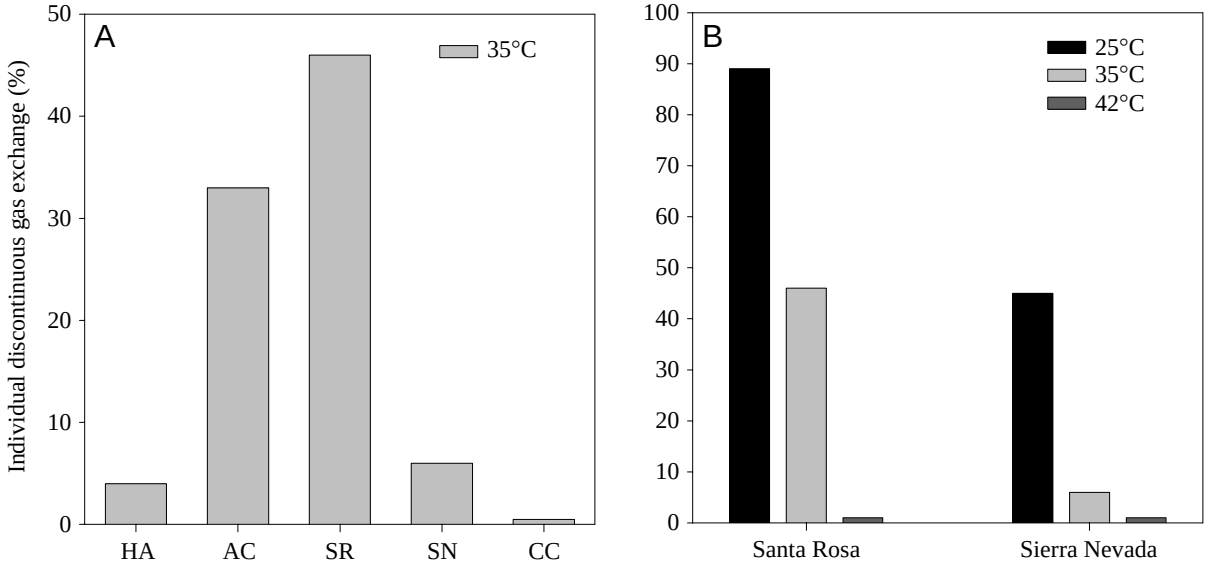

Fig. 6. (A) Frequency of occurrence of the discontinuous gas-exchange cycle (DGC) in populations of grasshoppers at 35 °C (N=116). The five populations are ordered by average rate of water loss, from low to high proceeding from left to right. No correlation could be made between water loss rate and the occurrence of discontinuous ventilation. HA, Hastings; AC, Angelo Coast; SR, Santa Rosa; SN, Sierra Nevada Aquatic Research Laboratory; CC, Crooked Creek. (B) Cessation of the DGC with increasing temperature. The occurrence of a DGC decreased dramatically with increasing temperature, for both populations. Only one individual displayed a DGC at the highest temperature, but both populations exhibited a relatively high frequency of DGC behavior at lower temperatures.

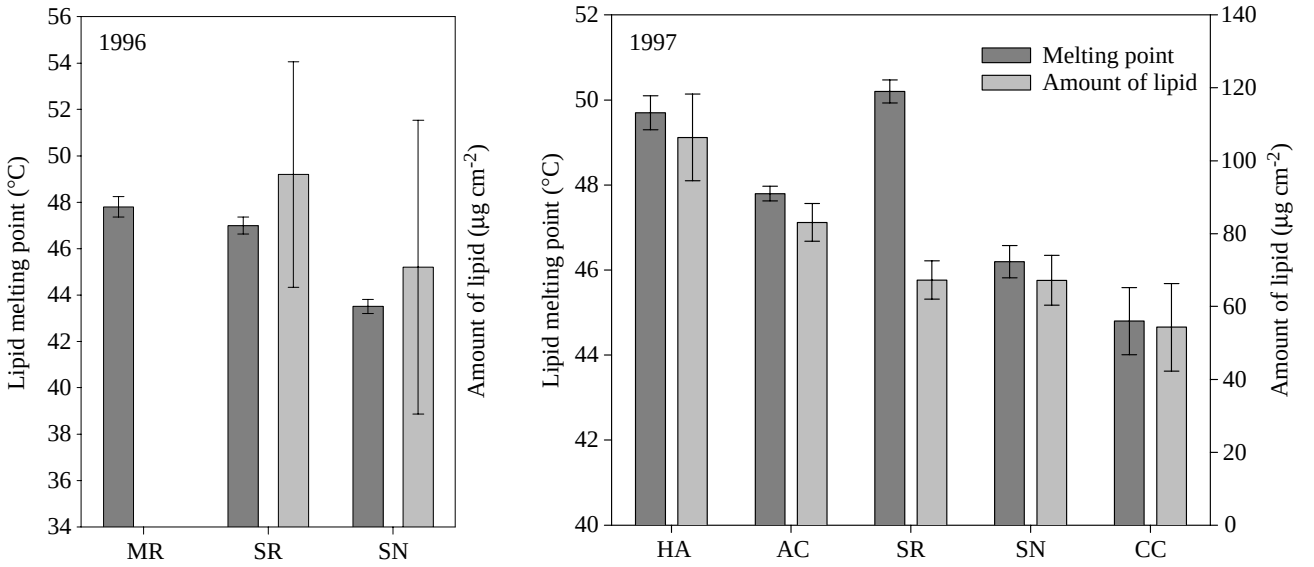

Fig. 7. Population differences in the amount of cuticular lipid and its melting point over 2 years. Populations are ordered along the axes by increasing water loss rate, with high-elevation, high-water-loss populations to the right (Crooked Creek and Sierra Nevada). Significant differences in melting point are evident between populations for both 1996 (N=114) and 1997 (N=116), but only the 1997 data show differences in the amount of lipid (ANCOVA, P<0.0001). Values are means ± S.E.M. MR, Motte Rimrock; HA, Hastings; AC, Angelo Coast; SR, Santa Rosa; SN, Sierra Nevada Aquatic Research Laboratory; CC, Crooked Creek.

completely at 42 °C, except in one individual. In addition, the frequency of discontinuous gas exchange was not correlated with altitude or with total WLR.

Cuticular lipid properties

Populations displayed a large variation in the amount of cuticular lipid and the mean melting point of the lipid mixtures (Fig. 7). Within each yearly data set, pairwise comparisons of lipid melting point between populations were highly significant (ANOVA, P<0.0001), with two exceptions: CC *versus* SN (P=0.091) and HA *versus* SR (0.273) in 1997. Although the amount of lipid followed a general trend of decreasing with altitude, only two comparisons were significantly different in 1997, HA *versus* SR (ANOVA, P<0.0001) and AC *versus* CC (ANOVA, P=0.002). The comparison between SN and SR in 1998 was also significant (ANOVA, P=0.033). Mean lipid melting point and the mean amount of lipid did not change significantly within a population over a 2 month period, although variation from year to year was observed (ANOVA, P<0.0001). Fig. 8 shows

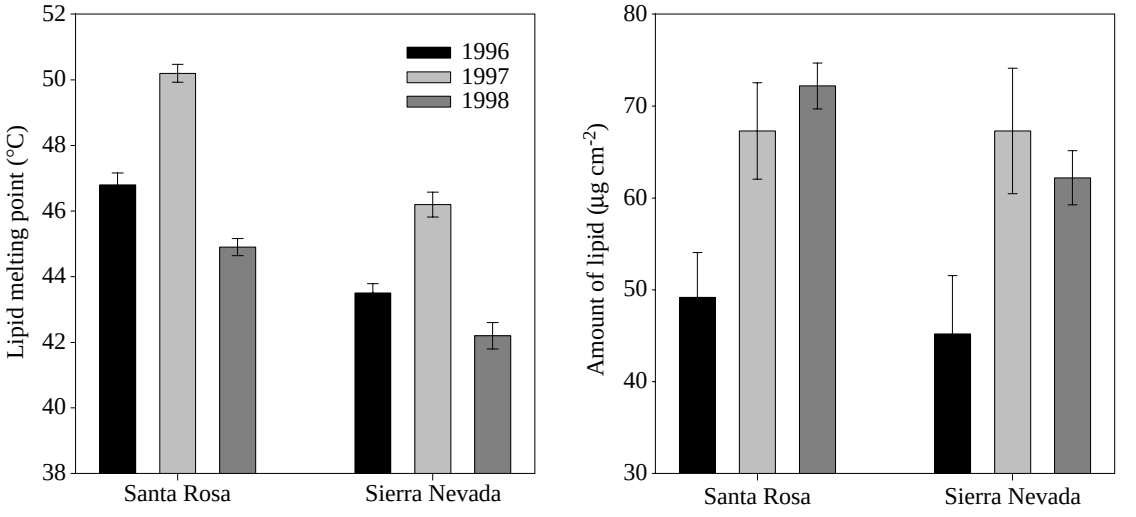

Fig. 8. Changes in lipid melting point and in the amount of lipid in two populations over a 3 year period (N=274). Significant year-to-year variation exists, possibly resulting from different climate patterns at the sites, with warmer temperatures inducing the production of lipids with higher melting point. Values are means ± S.E.M.

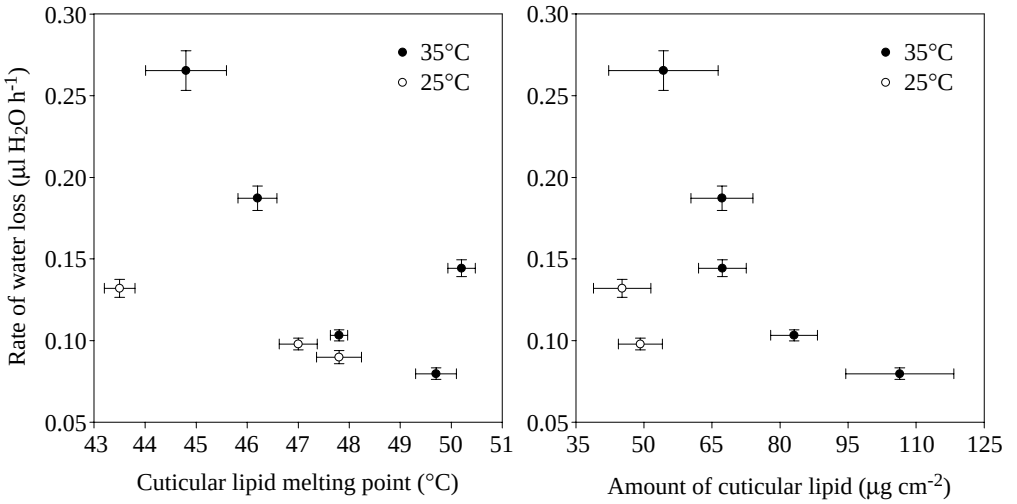

Fig. 9. The effect of cuticular lipid melting point and the amount of cuticular lipid on rates of water loss at two respirometry temperatures among populations of grasshoppers. Six different populations are represented (N=230), two (SR and SN) were measured at both temperatures. Populations with higher-melting-point lipids and greater amounts of surface lipids demonstrate lower rates of water loss. Data from 42 °C are not presented to simplify the figure, but display the same strong correlations (see Discussion). Values are means ± S.E.M. SR, Santa Rosa; SN, Sierra Nevada Aquatic Research Laboratory.

changes in the amount of lipid and melting point for two populations over 3 years.

Individual water loss rates were expressed per surface area (A), following the standard formula $A=12M^{2/3}$, where area is expressed in cm^2 and mass (M) in g (Edney, 1977). These rates of water loss were averaged for populations at each respirometry temperature. The mean population WLR was strongly negatively correlated with both the amount of lipid (r^2=1, 0.79) and its melting point (r^2=0.66, 0.99) (ANOVA, P<0.05) (Fig. 9).

Regressions of individual WLR expressed per surface area against the amount of lipid per surface area were generally not significant; only SN at 42 °C (P=0.041) and SR at 25 °C (P=0.014) showed negative correlations. At 42 °C, T_m and WLR per surface area were not correlated, at 35 °C trends were conflicting, with some populations (AC and SN, P<0.05) showing positive and some negative (HA, P=0.008) correlations. At 25 °C, all populations showed strong negative trends between WLR and T_m, and a negative correlation was seen in the SN population (P<0.001).

Discussion

Whole-insect water loss rates

The WLR measurements show large population differences between the low-elevation populations (AC, HA, MR and SR) and the two montane sites (CC and SN). The montane sites also seem to have a greater availability of water for the organisms, at least in the fields where these individuals were captured. The Sierra Nevada Aquatic Research Laboratory (SN) encompasses a creek that carries large amounts of snow-melt run-off during the summer, and the vegetation is considerably more lush than in any of the arid sites, although this is limited to several meadows at SN, and not to the general sage communities that surround the meadows. The Crooked Creek facility has several meadows, one of which borders a small pond, where the grasshoppers were collected. While several other species of grasshoppers were found in abundance at both CC and SN in the sage and more arid microhabitats, *M. sanguinipes* were found only in the meadows. They were consistently abundant at SN, but only a few individuals were found at CC.

The average air and soil temperatures during the growing season at each site are important abiotic factors to consider. Historical climate data from AC, HA and SN describe the locations as generally much warmer for the former two low-elevation sites. However, the alpine sites could be quite warm during typical summer afternoons, and solar radiation can be very strong at high altitude. Air temperatures easily reached 40 °C and soil temperature 50 °C at both CC and SN. Using the small temperature loggers, a more direct comparison may be made. Because of the logistics of retrieving and re-using the loggers, the greatest period of overlap for all sites was a 2 week period, 20–31 July 1997. Average 24 h air temperature was 9 °C at CC, 14 °C at SN and 20–22 °C at AC, HA and SR. Where both historical and logger data are available, the temperatures match to within 0.5 °C.

It is possible that the lower temperatures and greater availability of water act to reduce selection pressure on maintaining a low WLR. Nevertheless, field body temperature measurements clearly illustrate that, even in the high-elevation sites, grasshoppers can experience environmental temperatures close to or exceeding the melting point of their cuticular lipids. This underscores the importance of the lipid barrier, yet it is surprising that the T_b is so close to the reported population T_m, at least in SN individuals. From the relatively few data, it would appear that grasshoppers are selecting a preferred body temperature throughout the day. Interestingly, although WLR

is several-fold higher for SN and SR at 42 °C compared with 25 or 35 °C, the insects seem to use behavioral mechanisms to thermoregulate close to the high temperature, despite the pronounced water loss. It is likely that the metabolic advantage of feeding and digestion at higher temperatures may overcome the negative effects of increased water loss rates. I measured WLR under severely desiccating conditions; a flow rate of 100 ml min^{-1} and less than 0.5 % relative humidity are necessary for accurate sampling of water vapor. Under these conditions, WLR is greater than is likely in the field. Grasshoppers lost approximately 20 %, and in some cases 30 %, of their wet mass during a 24 h respirometry session, yet survived. At lower temperatures, individuals have survived for several days.

Effect of the discontinuous ventilation cycle on water loss rates

Three distinct phases were originally described for the discontinuous ventilation cycle (DVC), the open, closed and flutter phases. The open phase occurs when one or more of the spiracles is completely open (see review of spiracular control by Nikam and Khole, 1989; Harrison et al., 1995). The closed phase clearly prevents respiratory losses, but the flutter phase too is seen as critical. During the 'F' or flutter phase, air is believed to be taken into the spiracles but, because of the large inward convective flow, water would be prevented from escaping (Kestler, 1980).

Tests of the relationship between the DGC and water loss have produced conflicting results. Lighton et al. (1993a) found support for a DGC, yet Lighton and Garrigan (1995) have shown that, in ants, the F phase does not represent a water-saving mechanism. Noble-Nesbitt et al. (1995) found that cockroaches showed no significant effect of CO_2 release on WLR, and a variety of studies on flies have produced no correlations (Williams and Bradley, 1998; Williams et al., 1998). Most relevant to this current study, Hadley and Quinlan (1993) found that, in dehydrated grasshoppers, a DGC was absent or had no effect in individuals.

The amount of respiratory water loss relative to transcuticular water loss is variable in insects (see table in Hadley, 1994b), but ranges from less than 5 % to 20 %. When cuticular permeability is very low, respiratory water loss becomes a larger and more important component; when cuticular permeability is relatively high, it is argued that selection pressure for DGC behavior is relaxed because of its small effect on WLR (Lighton et al., 1993a,b; Hadley and Quinlan, 1993). In the ants studied, the DGC was seen as important when respiratory losses were approximately 13 % of the total, but not when losses where approximately 5 %. Cockroaches showed no effect of CO_2 on water loss, but respiratory WLR was only 4 % of the total (Noble-Nesbitt et al., 1995). The grasshoppers in the current study displayed respiratory losses greater than 20 %, yet DGC behavior vanished at high temperatures and was in no way correlated with reduced overall rates of water loss. The populations from the warmest locations, AC and HA, where cuticular losses are the lowest, show very little DGC behavior. This seems further to erode support for the importance of the DGC to respiratory savings, even where cuticular losses are lower.

Hadley (1994a) argues that a DGC is an unlikely mechanism for reducing respiratory water loss in natural populations because the DGC is disrupted by activity and occurs only at low temperatures. Grasshoppers would need to remain essentially motionless to achieve any benefits, yet they actively forage and thermoregulate during even the hottest periods of the day (Chappell, 1983; Whitman, 1987). Comparisons of WLR in individuals in the present study that displayed a DGC show that periods of DGC did not lead to significant savings over non-DGC periods. On the basis of these data, I can find no support for the role of the DGC in reducing respiratory or overall water loss rates in grasshoppers. The results of this study suggest that the occurrence of the DGC does not lead to water savings, even in insects showing respiratory losses. It is noted that, as the insect body temperature rises and approaches the melting point of the cuticular lipids, respiratory water loss becomes an even smaller fraction of overall water loss and the DGC is even less effective as a mechanism for reducing water loss.

Effects of cuticular lipid properties on water loss

There are many other routes for insect water loss other than respiratory, including through saliva and excretion (Edney, 1977), but the majority are transcuticular. As the primary barrier to water loss, the composition and amount of cuticular lipid figure largely in the determination of cuticular permeability. The composition of the lipids influences the melting point; compounds with a higher individual T_m, such as straight rather than branched alkanes, confer a higher T_m to the overall mixture (Gibbs, 1995). Both mixtures with a higher T_m and mixtures with a greater proportion of lipid should reduce cuticular permeability, and thus cuticular WLR (Lockey, 1988; Noble-Nesbitt, 1991). Fig. 9 shows the effect of increased melting point and larger amount of lipid in reducing water loss rates between populations at two respirometry temperatures; data for 42 °C are similar but are not shown in the figure. For all temperatures studied, strong negative correlations were found between T_m and WLR, and the amount of lipid and WLR. The large increase in WLR at 42 °C can be taken as evidence for the melting of the lipids, and the concomitant increase in permeability, as predicted by the molecular packing theory. A 35 % melting of the lipids has been shown dramatically to alter permeability (Rourke and Gibbs, 1999), and individuals from SN and SR have lipids that melt in the range 44–46 °C, although some melt at somewhat higher temperatures.

Other authors have detailed the composition of cuticular hydrocarbons for many species of insect (for reviews, see Blomquist et al., 1987; de Renobales et al., 1991; Noble-Nesbitt, 1991); in particular, much work has been carried out on the genus *Melanoplus* (Jackson, 1981). The composition can change in response to diet (Espelie et al., 1994) or acclimation to temperature (Gibbs and Mousseau, 1994). In the

present study, measurements made during the course of several months show that the lipids do not change significantly in the field within a population, and grasshoppers maintained in the laboratory were used in experiments before they had time to exhibit thermal acclimation, which in *M. sanguinipes* occurs after approximately 2 weeks. Independent tests of WLR also showed that they were highly repeatable and were stable in a population over the course of multiple field collections.

Changes in WLR resulting from lipid composition have been demonstrated (Toolson, 1982; Toolson and Kuper-Simbrón, 1989). In the current study, I directly measured individual T_m values from grasshopper lipid extracts, instead of inferring the melting point from their composition. Other studies have looked for correlations between T_m and WLR in flies, but have found none (Gibbs et al., 1997, 1998). Populations of laboratory-selected flies showed lower WLRs, but it could not be attributed to melting point. A further complication in the case of the *Drosophila* spp. studies is the role of cuticular hydrocarbons as sex pheromones (Chapman, 1998; Gibbs et al., 1998; Cobb and Jallon, 1990). The action of the pheromone may require a partially melted lipid layer or, because of the necessity of incorporating large amounts of the pheromone, T_m may be constrained by selection. No such complication is conceivable in *M. sanguinipes*, as no pheromone has ever been identified, although efforts were made to identify and characterize them.

Both the amount of lipid and its melting point appear to have a profound effect on the overall WLR in natural populations of grasshoppers. The SR population presents an interesting case study: WLRs are slightly higher than the lowest rates recorded for AC and HA populations, but are below those of the high-elevation populations. Santa Rosa individuals have among the lowest amount of lipid, but the highest T_m. All other populations have both high T_m and large amounts of lipid (AC and HA), or low T_m and low amounts of lipid (CC and SN). It is reasonable to ask why populations do not all simply produce lipids with high melting points and in large amounts. Populations do show significant variation in cuticular lipid components (B. C. Rourke, unpublished data). There are probably larger metabolic costs associated with synthesizing longer-chain hydrocarbons, one of the main high T_m constituents, but little is yet known about the cost of synthesizing and transporting lipids for deposition in the cuticular layer.

It is important to note that the cuticular lipid properties of the populations did not change significantly during collections several months apart because this could potentially confound the comparisons made among populations. However, year-to-year variation does exist, particularly in response to annual temperature fluctuations. Plasticity of lipid properties among populations has been demonstrated in response to thermal acclimation, although the response appears to be constrained by genotype as well (Gibbs and Mousseau, 1994).

Metabolic rate estimates

Carbon dioxide release has been used to estimate metabolic rates indirectly in insects (Lighton and Fielden, 1995; Williams et al., 1997, 1998; Williams and Bradley, 1998). It was critical to observe primarily the pattern of respiration and loss of water from the insect, particularly during spiracular opening and closing, and to note the discontinuous ventilation cycle, so rates of oxygen uptake were not measured. Moreover, the sensitivity of the equipment to carbon dioxide release is much greater. The possible confounding factor of varying respiratory quotients among populations or individuals is believed to be small because all grasshoppers were fed only romaine lettuce. A difference in metabolic storage or usage of foodstuffs is plausible, but not believed to contribute significantly to experimental error.

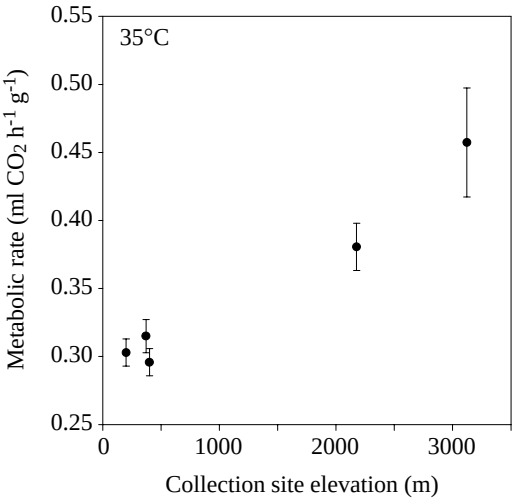

Fig. 10. Metabolic rates among low- and high-elevation populations measured at 35 °C. The two high-elevation populations (SN and CC) show substantially higher metabolic rates, which may act to compensate for shorter growing seasons and consistently lower air temperatures. Values are means ± S.E.M. (N=116). SN, Sierra Nevada Aquatic Research Laboratory; CC, Crooked Creek.

Fig. 10 shows the strong positive correlation between metabolic rate and elevation (y=5.108x+0.286, r^2=0.96). Increased metabolic rates in alpine populations of grasshoppers have been observed before (Ashby, 1997), and several authors have proposed that high mass-specific metabolic rates at elevation counter shorter growing seasons and colder temperatures (Chappell, 1982; Massion, 1983). However, Ashby (1997) found that, while populations seemed to have similar metabolic rates when environmental temperatures were taken into account, individuals from high-elevation populations tended to be smaller, which in itself would increase mass-specific values. I found that high-elevation populations of *M. sanguinipes* were actually significantly larger than low-elevation populations, and so mass-specific causes alone cannot be invoked. Values for Q_{10} did not vary between two populations, SN and SR, a result that fails to provide evidence for local adaptation. The SN and CC individuals may be larger as a result of the availability of a more lush vegetation or of more water, as discussed above.

Is there a conservation or compensation of metabolic rates?

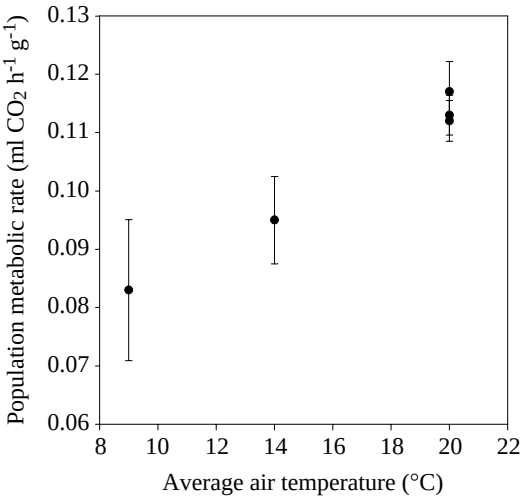

Fig. 11. Metabolic rates for five populations corrected for average July air temperature. The air temperatures are from data loggers placed at the sites during 1997, and the same Q_{10} was used for each population. Some error is introduced because the measured Q_{10} from two sample populations varied somewhat depending on the temperature range used. High-elevation populations, while possessing higher mass-specific metabolic rates, do not appear to be compensating completely for the lower ambient temperatures. Values are means ± S.E.M. (N=116).

Fig. 11 shows the mass-specific metabolic rates for each population, corrected for ambient temperature as measured during the 20–31 July period reported above. While high-elevation populations have higher mass-specific metabolic rates, they do not appear to compensate completely for the lower ambient temperatures. The average Q_{10} calculated from SN and SR populations over all three respirometry temperatures was used for all populations, which presents a potential source of error for those populations whose Q_{10} was not specifically measured. The actual temperature ranges over an entire growing season are also potentially misrepresented by the values used, and temperature during the day may be a more appropriate average than 24 h temperature, since much of the foraging and digestion occur during the diurnal periods.

I have shown that WLR varies among natural populations of grasshoppers and that the primary factors affecting WLR are the amount of cuticular lipid and its melting point. These data strongly support the classical theories of the importance of cuticular lipids to the permeability of the integument. Metabolic rate was also highly variable and strongly correlated with increasing elevation, but it is not a major factor in determining WLR. In contrast to arguments supporting the importance of a discontinuous gas-exchange cycle to respiratory water loss, this study finds no evidence for significant water savings through the discontinuous gas-exchange cycle.

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