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EFFECTS OF LIPID PHASE TRANSITIONS ON CUTICULAR PERMEABILITY: MODEL MEMBRANE AND *IN SITU* STUDIES

BRYAN C. ROURKE* AND ALLEN G. GIBBS‡

Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697, USA

*e-mail: bcrourke@uci.edu

‡Present address: Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

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Summary

The role of lipid physical properties in cuticular water loss was examined in model membranes and intact insects. In model experiments, pure hydrocarbons of known melting point (T_m) were applied to a membrane, and the effects of temperature on permeability were quantified. Arrhenius plots of permeability were biphasic, and transition temperatures for water loss (T_c) were similar to T_m. In grasshoppers *Melanoplus sanguinipes*, changes in cuticular water loss were measured using flow-through respirometry. Transition temperatures were determined and compared with T_m values of cuticular lipids, determined using Fourier transform infrared spectroscopy, for the same individuals. Individual variation in T_m was highly correlated with T_c, although T_m values were slightly higher than T_c values. Our results show that, in both intact insects and model membranes, lipid melting results in greatly increased cuticular permeability.

Key words: lipid, phase transition, permeability, cuticle, water loss, grasshopper, *Melanoplus sanguinipes.*

Introduction

Despite their small size and relatively large evaporative surface area, thousands of terrestrial insect species have successfully radiated into even the warmest, driest climates. The development of an impermeable cuticle is responsible for much of the ability of an insect to resist desiccation, although other strategies are evident, such as behavioral avoidance of low humidity and high temperature (Hadley, 1994). It has long been known that the primary barrier to cuticular water loss in insects is the epicuticular lipids (Wigglesworth, 1945). The species-specific composition of cuticular lipids has been elucidated for numerous insect taxa (Blomquist and Jackson, 1979; Hadley, 1981; Lockey, 1985, 1988; Blomquist et al., 1987; de Renobales et al., 1991) and is dominated by straight-chain, mono- and dimethyl branched hydrocarbons, wax esters and fatty acids. Differences in the relative abundance of certain lipid components have been demonstrated among congeneric species (Bartelt et al., 1986; Cobb and Jallon, 1990) and among populations of the same species (Gibbs et al., 1991; Ferveur et al., 1996).

Correlations between lipid composition and cuticular permeability have been observed on several occasions (Hadley, 1978; Toolson and Hadley, 1979; Toolson, 1982, 1984), but the exact relationship is unclear. A widely accepted model holds that cuticular permeability depends primarily on the physical properties of the surface lipids. This model was originally proposed to explain the phenomenon of critical temperatures for water loss (Wigglesworth, 1945). A common observation is that cuticular permeability to water remains fairly constant as the temperature is raised, but increases dramatically as a ‘critical’ or ‘transition’ temperature is reached (Wigglesworth, 1945; Lees, 1947; Loveridge, 1968; Hadley and Quinlan, 1989). This phenomenon can be explained by a molecular packing model in which the phase transition from a crystalline gel phase to a fluid state is accompanied by increased inter-molecular distance and reduced inter-molecular bonding. This theory states that the permeability of the wax layer to water is directly related to the physical properties of the surface lipids, particularly the melting point.

Several variations of this model have been proposed. Beament (1958, 1961, 1964) proposed the monolayer hypothesis, in which the water-proofing layer would consist of polar lipids, oriented at a specific angle in a layer one molecule thick. Lipid melting would change the orientation of the molecules making up the monolayer, resulting in the observed transition phenomenon by creating gaps through which water could pass. Several lines of evidence discredit this hypothesis: insect cuticular lipids are composed primarily of nonpolar alkanes and alkenes, and scanning electron microscopy has shown the deposition of lipids to be greater than the thickness of one molecule (Hadley, 1985; Lockey, 1988). Locke (1965), and later Davis (1974), instead hypothesized that gel–fluid phase transitions, occurring in the wax canals through which lipids are transported to the surface, were responsible. Hadley (1994) summarizes our current understanding, wherein the epicuticular lipid layer is the principal barrier to water loss, and

melting of lipids not only in the wax canals but over the entire surface of the cuticle allows water to pass more freely across the cuticle.

Despite the widespread acceptance of the lipid-melting model, a critical analysis of evidence supporting it reveals several difficulties. The most serious problem is the limited number of biophysical measurements of lipid properties. The biophysical methods used often do not work well on small samples made up of complex mixtures of compounds, as is characteristic of cuticular lipids (Gibbs and Crowe, 1991). In some cases (e.g. Davis, 1974), no measurements appear to have been performed, but instead the presence of a transition in water loss was itself taken as proof that the surface lipids had melted. In other work, transitions in cuticular permeability occurred at a temperature similar to that at which lipid properties changed, but only one species was examined (Toolson et al., 1979; Machin and Lampert, 1990). Thus, the correspondence between the critical temperature and a change in lipid properties could have been coincidental. When multiple species have been examined, they have included very distantly related taxa, which are likely to differ in many aspects of cuticular physiology in addition to their complement of cuticular lipids.

Many techniques have been used to measure cuticular permeability and changes in permeability with temperature, including gravimetric techniques and a variety of moisture-sensing devices (Loveridge, 1980; Hadley, 1994). The gravimetric methods have produced many useful data, but care must be taken to eliminate unstirred layers and changes in relative humidity surrounding the insect. While mass measurements are useful for estimating whole-insect water loss, it is difficult to apply this technique to measurements of cuticle sections or other membranes. Humidity sensors provide increased sensitivity and resolution, as well as the ability to investigate membranes and to distinguish between respiratory and cuticular water loss (Lighton, 1994). A potential problem with all of these studies is inter-individual variation in cuticular permeability and in the complement of surface lipids (Gibbs et al., 1991; Gibbs and Mousseau, 1994), which can obscure the actual relationship between these variables (Beament, 1958). Finally, several workers have noted the problems caused by visual estimation of critical temperatures, which are exacerbated by differences in how data are presented (Toolson, 1980; Machin and Lampert, 1989).

A number of technical advances have made it possible to re-examine the lipid-melting model. The coupling of flow-through respirometry with infrared sensing of water vapor allows precise measurement of transpiration, even in organisms as small as *Drosophila melanogaster* (Lighton, 1994; Williams and Bradley, 1998). Fourier transform infrared (FTIR) spectroscopy makes it possible to study lipid properties in cuticular lipids extracted from individual insects (Gibbs and Crowe, 1991). The present study combines model-membrane and whole-animal studies to investigate the role of the physical properties of cuticular lipids in determining permeability and is the first to measure both critical temperatures for water loss and lipid melting temperatures in the same individuals. In our model-membrane experiments, pure hydrocarbons were applied to a Gore-Tex membrane, and changes in permeability were observed with increasing temperature. Critical temperatures (T_c) were determined through curve-fitting to permeability data and were correlated with the melting points (T_m) of these compounds. We also used flow-through respirometry to measure T_c for individual grasshoppers (*Melanoplus sanguinipes*), and the T_m of surface lipids extracted from each individual was determined using FTIR spectroscopy. We found strong positive correlations between T_c and T_m in both sets of experiments. More importantly, T_c and T_m values were nearly identical, providing strong evidence for the lipid-melting hypothesis.

Materials and methods

Grasshopper collection and husbandry

Adult grasshoppers (*Melanoplus sanguinipes* Orthoptera: Acrididae) were collected during July 1997 from four locations in California, USA (Table 1). Previous work (Gibbs et al., 1991; Gibbs and Mousseau, 1994) has demonstrated substantial variability in T_m for surface lipids among and within Californian populations of *M. sanguinipes*. Grasshoppers were transported to Irvine and maintained for several weeks on a diet of fresh romaine lettuce *ad libitum.* They were held in individual cages at 25 °C, on a 14 h:10 h L:D photoperiod.

Model-membrane apparatus

An acrylic sample holder was constructed following a schematic diagram provided by N. F. Hadley and M. C. Quinlan (Fig. 1). A Gore-Tex cardiac patch (W. L. Gore Medical, porosity 17 μm) approximately 1.5 cm in diameter was placed between the two halves of the central holder and was sealed in place between two silicone gaskets. The untreated membrane allowed water to move freely across the 2 mm diameter aperture. To coat the membrane, pure hydrocarbons of known melting point (Aldrich Chemicals) were dissolved in hexane and applied to the membrane. The lower side of the apparatus was filled with water, and the apparatus was checked visually for leaks. We also blew air into the sealed and submerged membrane apparatus and checked for the presence of bubbles on the aqueous side, which would indicate that air was leaking across the membrane or around the gaskets.

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
SNARL	37°36'51"N	118°49'47"W	2177
Hastings	36°12'30"N	121°33'30"W	144–293
Santa Rosa	33°48'45"N	117°15'30"W	370

SNARL, Sierra Nevada Aquatic Research Laboratory.

Fig. 1. Model-membrane apparatus, shown partially unassembled. All three sections are solid acrylic disks with hollowed-out core sections. The upper and lower disks are drilled to accept gas-tight copper tubing connectors. Air entered and exited the apparatus only through the top two ports, and water circulated only through the lower two ports. The membrane was held in the center section by silicone gaskets, which were in turn held in place by a large plastic retaining ring. The upper surface of the membrane was therefore exposed to dry air blown through the ports in the upper section, and the lower surface of the membrane was in contact with the water circulating within the lower section of the apparatus.

The apparatus was submerged in a temperature-controlled water bath, and dry air was blown over the surface of the membrane at 100 ml min^{-1}. The lower half was open to the water bath, while the upper portion was arranged to allow one-way air flow from the respirometer pump. We measured sample temperature using a thermocouple sealed in place just above the model membrane. Water vapor from below the membrane could pass into the upper chamber only by traversing the membrane-covered aperture into the air stream flowing over the top surface of the membrane. Data were recorded using a dew-point hygrometer (EdgeTech, Milford, Massachusetts, USA) connected to a computer-based data-acquisition system (Sable Systems, Henderson, Nevada, USA). Water vapor passing through the membrane was quantified as the temperature was increased from 25 to 60 °C at a heating rate of approximately 1 °C min^{-1}. Five pure straight-chain alkanes (carbon lengths C_{19}–C_{23}, T_m=33–49 °C) were used in these experiments. We attempted to use other alkanes, particularly C_{24}, C_{25} and C_{26}, but were unsuccessful in sealing the membrane sufficiently. When evaporated from hexane, the crystalline structure of these compounds is flaky and they did not adhere well to the membrane. Moreover, the range of melting temperatures provided by the C_{19}–C_{23} alkanes is within the biologically relevant range of T_m values for the surface lipids of *M. sanguinipes* and other species, such as houseflies and *Drosophila melanogaster* (Gibbs et al., 1995, 1998). The melting point for C_{23} alkane is approximately 50 °C, which is near the upper thermal limits for many insects.

Water loss from grasshoppers

Rates of water loss from adult grasshoppers were measured using flow-through respirometry in a temperature-controlled cabinet (Sable Systems). Room air was scrubbed of carbon dioxide and water vapor, then pumped at 100 ml min^{-1} through a 5 ml glass chamber containing the grasshopper; sample air was then passed to an infrared carbon dioxide and water analyzer (Licor Instruments, model LI-6262). Rates of water loss and carbon dioxide release were measured using two protocols. In the first, the temperature was increased from 25 to 50 °C, in intervals of approximately 2 °C, and the average rates of CO_2 and water loss were recorded for 20 min at each temperature. A baseline was recorded from an empty respirometry chamber after the temperature of the cabinet had stabilized at the next level. Temperature had a negligible effect on baseline values for CO_2 and water vapor, compared with loss rates for grasshoppers. Alternatively, the temperature was increased continuously from 27 to 60 °C. This ramping protocol yielded a sigmoidal heating curve, with rates of heating decreasing as the cabinet reached the upper setpoint. Data were analyzed from the middle portion of the protocol only, where the rate of heating was linear and approximately 0.5 °C min^{-1}.

Individual grasshoppers from the four populations were assigned to four treatment groups. Approximately half the grasshoppers were alive during respirometry; the others were placed in a vial containing potassium cyanide for approximately 3 h before measurement. Individuals treated with cyanide demonstrated no active gas exchange and were therefore considered dead. The live and cyanide-treated individuals were further divided into continuous and discrete sampling protocols, as outlined above. This gave a total of four possible treatments: live-continuous recording, live-discrete recording, dead-continuous recording and dead-discrete recording. Individuals were fasted for 24 h before measurements were made.

Determination of water-loss breakpoint (T_c)

All data were first corrected for the vapor pressure deficit of water and subsequently displayed as Arrhenius plots. Respirometry data were analyzed using the procedure of Duggleby and Ward (1991), which was developed to characterize plots in which two linear segments best approximate data around a point of marked slope change. This curve-fitting procedure uses a least-squares method to estimate the coordinates of the transition point and the slopes of the lines on either side. The function was applied using SigmaPlot for Windows (Jandel Scientific, version 2.0), and the breakpoint for each individual was converted to °C.

Determination of cuticular lipid melting point (T_m)

Grasshoppers were removed from the respirometry chamber at the completion of the run and stored at –70 °C. Surface lipids were extracted from each individual by immersion in hexane for 15 min at room temperature (23–25 °C), and the hexane was then evaporated under nitrogen gas. Lipid samples were later

redissolved in hexane, evaporated onto CaF_2 windows, and placed in a temperature-controlled sample holder for Fourier transform infrared (FTIR) spectroscopy (Gibbs and Crowe, 1991). We used the -CH_2- symmetrical vibrational stretching frequency as our index of lipid melting. A sigmoidal function was fitted to the FTIR data using SigmaPlot, and the midpoint between the upper and lower asymptotes of the curve was defined as the melting point (T_m).

Results

Model-membrane experiments

Arrhenius plots of water flux through hydrocarbon-coated membranes were biphasic, with a steeper slope at higher temperatures (Fig. 2). Fig. 3 depicts the relationship between T_c and T_m for five hydrocarbons (C_{19}–C_{23}). Transition temperatures for water loss and lipid T_m values were highly correlated (r^2=0.91, P<0.001) and were within a few degrees of each other for all five compounds.

Water loss from grasshoppers

Arrhenius plots of transpiration data for intact grasshoppers were similar to those obtained in the model-membrane experiments (Fig. 4). Fourteen live grasshoppers were assayed for T_c, including seven continuous recordings and seven discrete ones (Fig. 5A). An analysis of variance revealed no heterogeneity in slopes (F-ratio=0.04, P>0.845), permitting an analysis of covariance (ANCOVA). This revealed no treatment effect for either discrete or continuous recording, so the data were pooled for regression analysis. The regression between T_c and T_m was highly significant (r^2=0.779, P<0.0001):

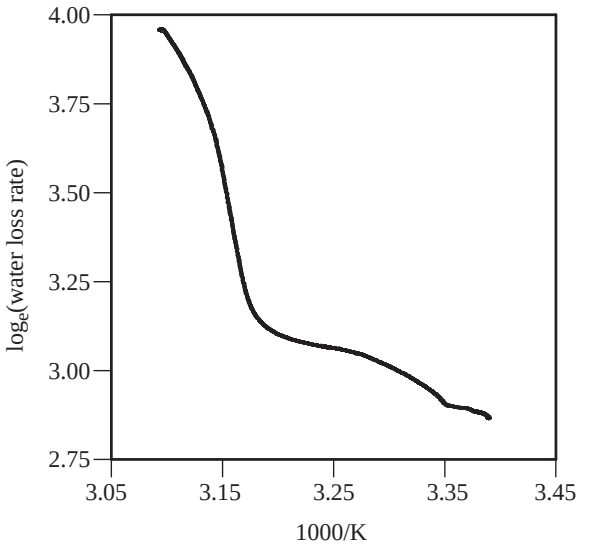

Fig. 2. Effects of temperature on the permeability of a hydrocarbon-coated Gore-Tex membrane. Sample data are presented for one of the hydrocarbons used, in this example *n*-docosane (melting point, T_m=44 °C). In this Arrhenius plot, temperature increases from right to left along the ordinate. Approximately 6000 data points are plotted, and no smoothed curve has been drawn through the points. Rates of water loss were measured in μl h^{-1}.

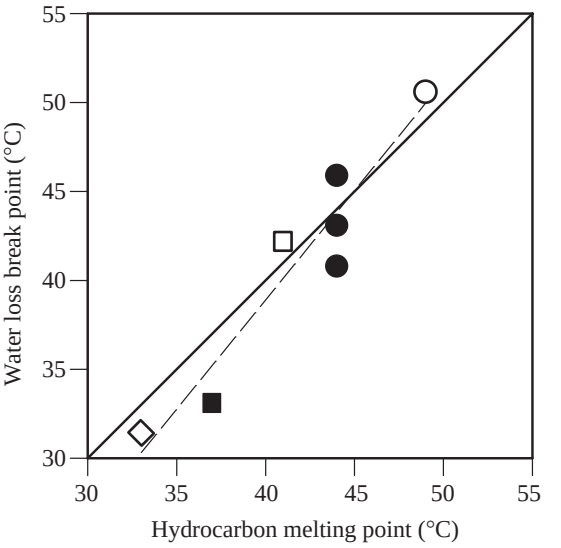

Fig. 3. Correlation between transition temperature for water loss and melting point for five pure hydrocarbons. One compound (*n*-docosane) was tested on three occasions. Pure, straight-chain alkanes (chain length C_{19}–C_{23}) were used and ranged in melting point from 33 °C (C_{19}) to 49 °C (C_{23}). The diagonal represents the line of equality between alkane melting temperature and alkane transition temperature. The dashed line is a regression through the data: y=1.22x–10.19 (r^2=0.91, P<0.001).

T_c=(1.04±0.16)T_m–4.35±7.38 (means ± S.E.M.). For a given individual, T_c was generally approximately 2 °C below T_m.

Twelve cyanide-treated grasshoppers were assayed for T_c. In an early experiment, recordings were made from one individual at discrete 20 min intervals. Because the continuous-ramping protocol provided greater resolution, and because there was no treatment effect of recording type evident in data from the live grasshoppers, recordings were made from all other cyanide-treated individuals using the continuous protocol. A regression of transition temperature on T_m (Fig. 5B) was highly significant (r^2=0.50, P<0.009) with a slope of 0.98±0.31 (mean ± S.E.M.). In both live and dead individuals, the break point for water loss was slightly lower than would be expected from the lipid melting point.

FTIR spectroscopy

Representative melting curves for lipids extracted from these specimens are shown in Fig. 6. Surface lipids began melting at 30–40 °C and were completely melted above 55 °C. In addition to T_m (the midpoint of the lipid phase transition), we calculated the percentage of lipid melted at the transition temperature for water loss for each individual. The minima and maxima of the individual melting curves (values as wave number, cm^{-1}) were treated as 0 and 100 % melted, respectively. The corresponding wave number for the critical temperature was determined by linear interpolation between the two closest points from the melting curve. Using this measure, the average percentage of lipids melted at the transition temperature was 34.5±3.3 % (mean ± S.E.M., N=26).

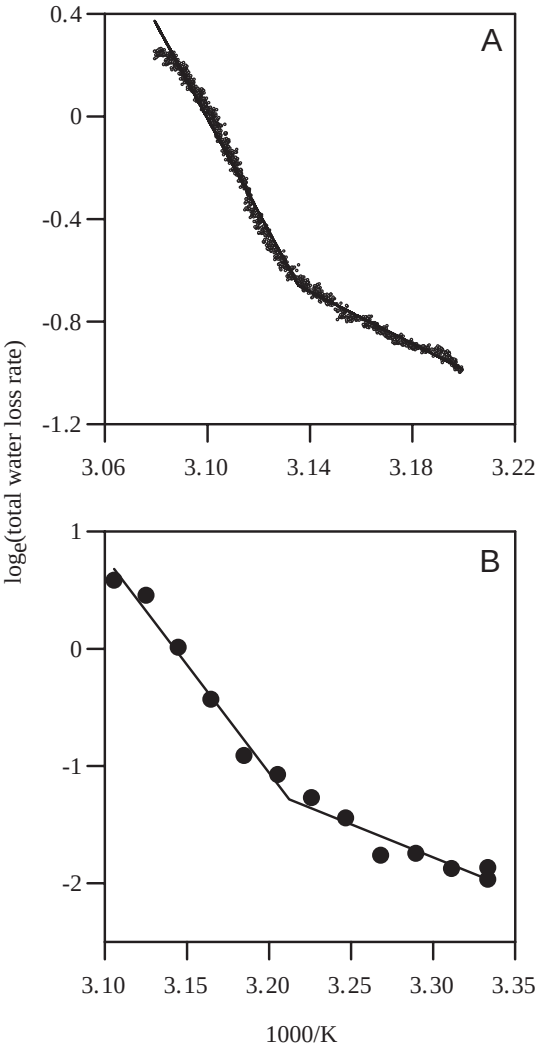

Fig. 4. Effects of temperature on rates of water loss (measured in $\mu l\,h^{-1}\,mmHg^{-1}$; 1 mmHg=0.133 kPa) from intact grasshoppers. (A) Continuous protocol on cyanide-treated insects. Data were recorded every 2 s. (B) Discrete protocol on live insects. Each point represents the average loss over a 20 min period. The transition temperature for each curve was calculated as the intersection of two best-fitting lines (see Materials and methods).

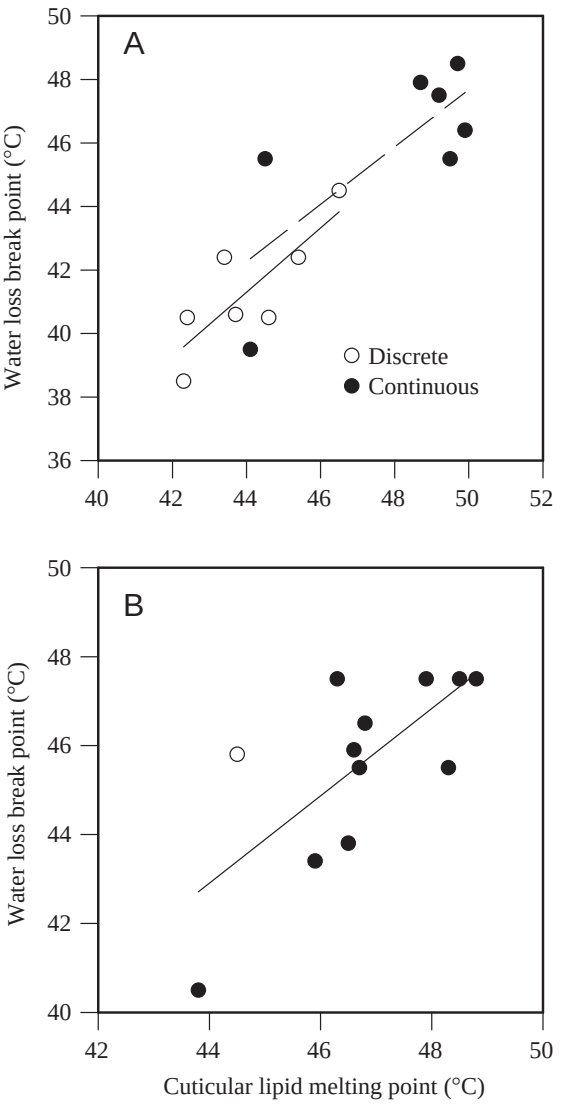

Fig. 5. Comparison of the transition temperature for water loss (T_c) with cuticular lipid melting point (T_m) for individual grasshoppers. (A) Live grasshoppers. (B) Grasshoppers killed with cyanide vapor. Data are from both continuous recordings (filled circles) and discrete protocols (open circles). Note that the water loss break point is slightly lower than would be expected from the lipid melting point. See text for details of regression lines.

Discussion

The transition phenomenon is evident in a variety of insects (Wigglesworth, 1945), ticks (Lees, 1947; Davis, 1974), spiders (Hadley and Quinlan, 1989) and other arthropods. The hypothesis that melting of the surface lipids is responsible enjoys a long history in the literature (Ramsay, 1935; Wigglesworth, 1945), but a clear demonstration has not been provided. With recent advances in techniques, we can measure T_c and T_m values with greatly improved precision in individual specimens. Our use of individual variation in lipid properties within a single species minimizes potential problems associated with comparing phylogenetically diverse arthropods. By combining the model-membrane and *in situ* experiments, we were able to perform a rigorous test of the lipid-melting hypothesis.

Our model-membrane system is clearly much simpler in structure than an arthropod cuticle, but it provides an important test of the lipid-melting model. The only parameter varied in these experiments was the type of hydrocarbon used. If we had not observed the transition temperature phenomenon at all or if T_c values had not corresponded closely with T_m values for the pure hydrocarbons, then our results would have provided strong evidence against this model. The near identity of these two parameters, over a range of 16 °C, provides support for the lipid-melting hypothesis. The slope of a regression between T_c and T_m was 1.23±0.17, mean ± S.E.M., N=7) and was not significantly different from 1 (P<0.24), as one would predict from the model.

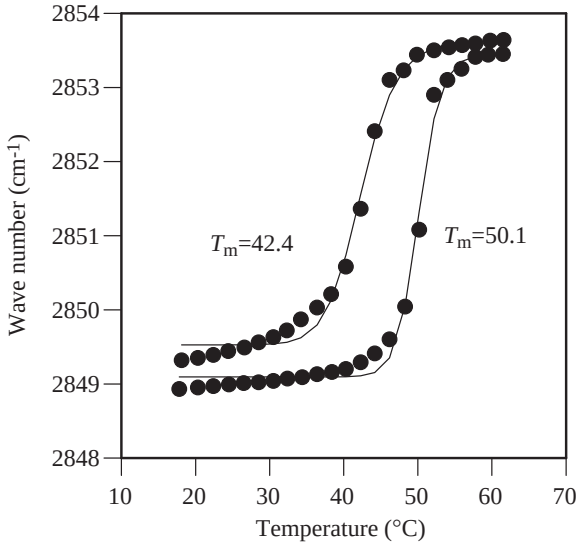

Fig. 6. Representative melting curves for cuticular lipids extracted from grasshoppers. The abcissa indicates the vibrational frequency (wave number) of $–CH_2$- symmetrical stretching motions (Gibbs and Crowe, 1991). Data and fitted curves for two individuals are presented. The melting point (T_m) is defined as the midpoint of the transition.

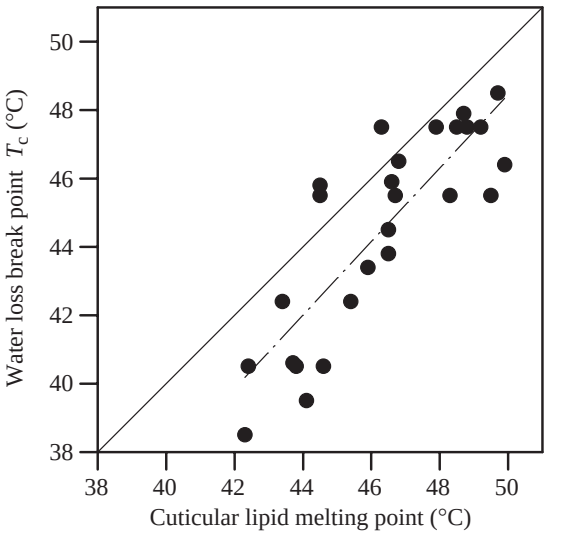

Fig. 7. Comparison of transition temperature for water loss (T_c) and cuticular lipid melting point (T_m) for all grasshoppers and all experimental protocols. A line of equality (solid line) and a regression through the data (dashed and dotted line) are drawn. The regression equation is $y=1.07x+5.23$ ($r^2=0.699$, $P<0.62$).

As previously observed by many investigators, a breakpoint or critical temperature for water loss was observed in both live and dead grasshoppers. Here, the variation afforded by individual variation in T_m values of *M. sanguinipes* removes possible artefacts resulting from the use of distantly related species to obtain varying T_c values. A powerful advantage of the present study is the ability to compare simultaneously the observed transition temperatures with the melting points of surface lipids from the same individuals. We found that the two temperatures were highly correlated, and nearly identical, again supporting the lipid-melting model. When all data were pooled, the slope of the regression between T_c and T_m was not significantly different from 1 (1.07±0.14, mean ± S.E.M., $P<0.62$) (Fig. 7).

It is important to note that T_c values were consistently lower than T_m for the same individuals (Fig. 7). The average difference was 1.8 °C and was highly significant ($P<10^{-5}$, two-tailed *t*-test). Lipid extracts from *M. sanguinipes* are complex mixtures of 20 or more wax esters and long-chain hydrocarbons, which melt over a range of several degrees. For our analysis, we used the midpoint of the melting range, as indicated by FTIR spectroscopy, but it is clear that a lower percentage of melting in the mixture can change molecular packing sufficiently to induce greater water loss. On average, the T_c occurred when 35 % of the mixture had melted, suggesting that the surface lipids do not need to be completely melted for an increase in permeability to occur. Alternatively, regional variation in lipid properties may exist, so that different areas of the cuticle increase in permeability at different temperatures.

An important concern in experiments using intact grasshoppers is the significance of non-cuticular routes for water loss. Insects also lose water through the spiracles during respiration and *via* the feces and saliva. The latter two routes of water loss may be eliminated from our consideration because grasshoppers were not observed to defecate during the recording, nor were they feeding. At low temperatures, live individuals exhibited a discontinuous pattern of CO_2 release, as has been demonstrated in other species of grasshopper (Hadley and Quinlan, 1993). An increase in water loss through the spiracles was associated with gas exchange, but total water loss never increased by more than 20 % when the spiracles opened, and the increase was usually less than 10 %. At higher temperatures, ventilation became disorganized, and it was impossible to distinguish respiratory from cuticular water loss. However, CO_2 release increased less rapidly with temperature than did water loss, so we conclude that respiratory water losses comprised a small and decreasing proportion of overall water loss as temperature increased, and that most of the increase in transpiration was caused by increased cuticular permeability. Live and cyanide-killed individuals lost water at similar rates, also supporting the conclusion that the transition phenomenon is not an artefact of increased respiratory water loss.

It is important to distinguish our experiments from those in which inter-specific or intra-specific variation in lipid composition has been correlated with differences in rates of water loss (Hadley, 1978; Toolson, 1982, 1984). These have been cited as supporting the lipid-melting model because species or individuals having longer chain-length hydrocarbons (which should melt at higher temperatures) tend to lose water less rapidly. However, rates of water loss were measured at only one temperature, and neither T_c nor T_m was

measured, so it is impossible to know what physical state the surface lipids were in. We note that longer chain lengths do not necessarily result in higher T_m values because of the effects of unsaturation and methyl-branching on hydrocarbon properties (Gibbs, 1995; Gibbs et al., 1995, 1998). Correlations between lipid composition and water loss may be due to differences in lipid properties, but could also reflect differences in the amount of surface lipid. Our experiments were not designed to examine how lipid amount affects rates of water loss in different insects; we were interested in how temperature affects lipid properties and transpiration from a given individual. We are separately investigating whether variation in T_m affects cuticular permeability at temperatures below T_m (B. C. Rourke, unpublished data).

Because we used field-caught grasshoppers, which were then maintained in the laboratory, we cannot determine the extent to which individual variation in T_m was caused by genetic or environmental factors (Gibbs et al., 1991; Gibbs and Mousseau, 1994). However, our results do suggest that this variation can be ecologically relevant in nature. We calculated that the T_c occurred when cuticular lipids were approximately one-third melted. Body temperatures of *M. sanguinipes* in the field are typically approximately 40 °C and can reach 43 °C (Chappell, 1983; B. C. Rourke, unpublished data). Thus, significant lipid melting can occur in nature and may cause increased rates of water loss (Fig. 7). Our findings support the hypothesis that natural selection has acted to increase T_m and decrease water loss in populations from lower latitudes (Gibbs et al., 1991).

To summarize, we have subjected the lipid-melting model for cuticular permeability to a rigorous, statistical test. In both model-membrane and whole-organism experiments, T_c and T_m are highly correlated. More importantly, they are nearly identical, and regression slopes are statistically indistinguishable from 1. We conclude that the model is generally correct, but that complete melting of the surface lipids is not required to cause a transition in cuticular permeability. Molecular packing is sufficiently disrupted when the lipids are approximately one-third melted, a degree of melting that is ecologically relevant in the case of *M. sanguinipes*.

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