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Cuticular lipids and water balance

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Epicuticular lipids serve many roles in insects and other terrestrial arthropods (see other chapters in this volume), but the first to be recognized was as a barrier to transpiration through the surface of the animal. Surface-area to volume ratios increase as size decreases, so that smaller animals become increasing susceptible to dehydration. Kühnelt (1928; cited by Wigglesworth, 1933) noted the presence of hydrophobic substances on the insect cuticle and proposed that these reduce water loss. Their importance in water conservation is made apparent by the fact that even a brief treatment with organic solvents to remove surface lipids can result in water-loss rates increasing 10–100 fold (Hadley, 1994). On the other hand, water can be lost via other routes. These losses can be substantial (e.g. blood-sucking insects excrete huge amounts of fluid after a meal). Thus, under certain conditions, cuticular waterproofing may be a minor part of the overall water budget.

We address several issues in this chapter. First, is cuticular water loss significant? The importance of spiracular control and discontinuous gas exchange in insect water balance has been challenged in recent years, leading to a need to reassess insect water budgets in general. Second, if cuticular transpiration is important for the overall water budget of insects, how does variation in surface lipid composition affect transpiration through the cuticle? We will discuss the relationships between lipid composition, lipid physical properties, and cuticular permeability. Third, what is the biophysical explanation for the critical temperature phenomenon, a rapid increase in water loss at high temperatures? We consider both experimental results and a few theoretical issues that address this question. Finally, do other cuticular structures besides lipids significantly affect insect water budgets? Our goal is both to describe what we do know, as well as to indicate gaps in our knowledge of cuticular function.

Is cuticular permeability important? Cuticular and respiratory water loss

Consideration of the waterproofing function of cuticular lipids first requires an assessment of cuticular transpiration relative to the overall water budget. The fact that organismal water loss rates increase greatly when surface lipids are removed does not necessarily mean that increased cuticular permeability is responsible. Insects can lose water by transpiration through the cuticle, by evaporation from the tracheal system through open spiracles, and by

various excretions (oral secretions, feces, eggs, and even "sweat"; Hadley, 1994; Toolson and Hadley, 1987). Respiratory water loss can be particularly important. Many insects exhibit cyclic breathing patterns, in which spiracles are held closed for some period, then open to allow release of accumulated carbon dioxide. The textbook explanation for this phenomenon is that it is an important water conservation mechanism. However, a number of recent studies have challenged this idea, alternative hypotheses have been put forward, and insect respiration has become an active and sometimes contentious field (see Chown, 2002; Chown *et al*., 2006; Quinlan and Gibbs, 2006 for more discussion).

In the context of this chapter, respiratory behavior is important only to the extent that it affects our understanding of cuticular water loss. Organic solvents tend to kill insects, causing spiracular control to be lost. It has often been assumed that spiracles close upon death, and therefore water loss from dead animals reflects cuticular transpiration, but water loss rates can increase after death (e.g., they approximately double in *Drosophila melanogaster*; A. G. Gibbs, unpublished observations), suggesting that spiracles may open in some species. Thus, it is conceivable that solvent-extracted animals lose significant amounts of water via the spiracles after death.

With flow-through respirometry, water loss associated with spiracular opening can be detected easily in large enough insects. Water-loss rates can more than double when spiracles open (e.g., Lighton *et al*., 1993; Figure 6.1). In *Manduca* pupae, opening of a single "master" spiracle is sufficient for whole-organism gas exchange (Slama, 1988). With few exceptions (e.g., Duncan and Byrne, 2002; Byrne and Duncan, 2003), we do not know how many or which spiracles open to allow gas exchange (and respiratory water loss). Based on the *Manduca* example, however, theoretically a solvent-extracted insect with 10 spiracles could lose water 10 times faster simply because of loss of spiracular control.

Because of issues regarding respiratory control, water loss measurements in live animals are preferable to those using dead animals, particularly when experimental techniques allow cuticular, respiratory and other components of water loss to be distinguished from each other. This is relatively easy when insects exhibit discontinuous gas exchange (Hadley and Quinlan, 1993); when they do not, alternative methods have been developed (Gibbs and Johnson 2004; Lighton *et al*., 2004). Such studies have generally shown that, while respiratory water loss constitutes a significant fraction of the water budget (especially in active insects), cuticular water loss generally accounts for >80% of overall water loss (Hadley, 1994; Chown *et al*., 2006; Quinlan and Gibbs, 2006). Figure 6.2 illustrates the potential importance of cuticular transpiration for overall water balance. In this example, differences in cuticular water loss accounted for 97% of inter-individual variation in total water loss (Johnson and Gibbs, 2004).

We have, to some extent, set up a straw man in this section, but it is important to recognize that most studies have not distinguished cuticular transpiration from other components of the overall water budget. This is probably not a serious problem for work with inactive insects; it may be in other cases. The permeability of the cuticle to water is clearly an important aspect of insect water balance, but rigorous analysis requires a good quantitative understanding of cuticular and other routes for water loss. Below, we first discuss the role

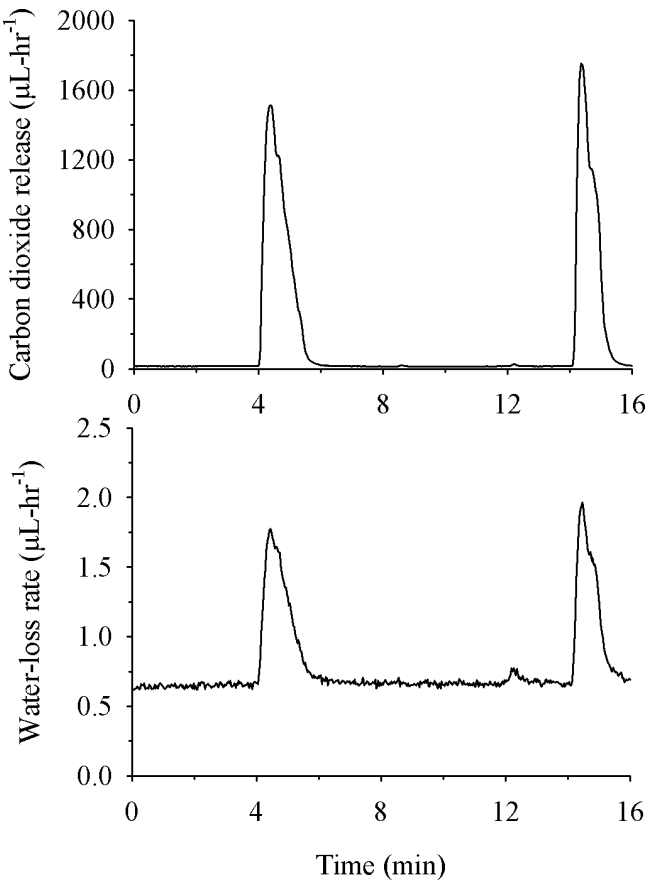

Figure 6.1 Discontinuous gas exchange in a grasshopper, *Trimerotropis pallidipennis*. Note that total water loss more than doubles when the spiracles open to allow gas exchange. In this case, cuticular transpiration is easily estimated by measuring water loss when CO_2 release is negligible.

of surface lipids in waterproofing the cuticle, then other aspects of cuticle architecture that may also affect water loss.

Correlations between cuticular lipids and water loss rates

The composition of cuticular lipids varies at all levels of organization in insects, from among species to within individuals. The amount of cuticular lipid can also vary substantially. For example, wax blooms of desert tenebrionid beetles are associated with reduced water loss (Hadley, 1994). High densities of wax may also serve to reduce heat load by reflecting solar radiation (Hadley, 1994) or to deter predators (Eigenbrode and Espelie, 1995); thus, it cannot be assumed that water conservation is the primary function of wax

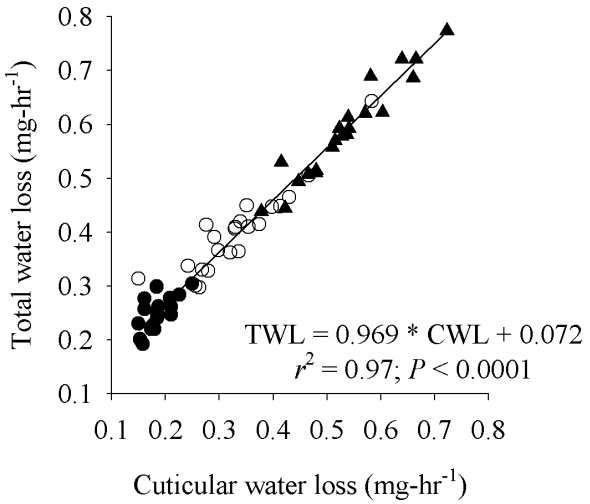

Figure 6.2 Correlation between cuticular water loss and total water loss in female ants, *Pogonomyrmex barbatus*. Differences in cuticular transpiration account for 97% of the variation in total water loss. Different symbols indicate differences in mating status. From Johnson and Gibbs (2004); used with permission.

blooms. It should be noted that blooms often do not form a homogeneous lipid layer across the cuticle (Nelson *et al*., 2000), which may result in varying permeability from one region to another. The effects of spatial heterogeneity on cuticular transpiration have received very little attention (e.g., Hadley and Quinlan, 1987; Toolson and Hadley, 1987; Machin and Lampert, 1989).

A number of studies have compared cuticular lipids and water-loss rates among related species (e.g. Hadley, 1978; Hadley and Schultz, 1987). Much of this work was done before the importance of accounting for phylogenetic relationships in interspecific comparisons was widely recognized (Felsenstein 1985; Garland and Adolph 1994; see also Chapter 7, this book). Intraspecific studies reduce phylogenetic concerns (although one still has to consider genetic relationships between populations) and should provide a better understanding of how changes in lipid composition affect cuticular transpiration.

Intraspecific variation in water-loss rates has often been associated with differences in cuticular lipids (e.g., Toolson and Hadley, 1979; Toolson, 1984, Johnson and Gibbs, 2004). At the population level, grasshoppers (*Melanopus sanguinipes*) from lower latitudes lose water slowly and have surface lipids with higher melting (Rourke, 2000), and long-term maintenance of *Drosophila* populations in culture affects both water loss and HC composition (Toolson and Kuper-Simbrón, 1989). Summer-collected scorpions and tenebrionid beetles have lower water-loss rates than fall/winter-collected animals, as well as a different complement of cuticular hydrocarbons (Hadley, 1977; Toolson and Hadley, 1979; see also Chapter 19). Thermal acclimation to higher temperatures in the laboratory causes similar changes (Hadley, 1977; Toolson, 1982), suggesting that seasonal temperature changes in

nature are responsible for both lipid variation and differences in water loss rates. It should be noted, however, that climatic changes are not the only factors affecting surface waxes. Hydrocarbon composition of *Drosophila mojavensis*, for example is affected by host plant (Stennett and Etges, 1997), and, in desert harvester ants, HC composition and water loss rate are correlated with queen mating status (Johnson and Gibbs, 2004).

The phase transition model for cuticular permeability

What makes a good cuticular waterproofing barrier? Is it simply enough to have a hydrophobic layer, or do lipids differ in their ability to reduce transpiration? Ramsay (1935) proposed the first mechanistic hypothesis, beyond the mere fact of hydrophobicity, for the function of cuticular waxes. An "incautious use of a tap" (Maddrell, 1990) splattered one of Ramsay's cockroaches (*Periplaneta americana*) with water. Ramsay noticed that beads of water on the surface of the cockroach evaporated much more slowly than drops on the lab bench. After further investigation, he concluded that lipids from the cuticle formed a hydrophobic coating around the droplets that reduced evaporation. He also noted that the properties of this coating were highly dependent upon temperature, with evaporative water loss increasing very rapidly above 30°C. After microscopic observations, Ramsay (1935) proposed that the lipids undergo a phase change at 30°C from a solid, relatively impermeable state to a melted, permeable condition.

Ramsay (1935) had the good fortune to be working with an animal with an unusual complement of cuticular lipids. The surface lipids of cockroaches include high levels of relatively polar compounds (by the standards of other insects), including alcohols, ketones, etc. (Schal *et al*., 1990; Buckner, 1993, see also Buckner's Chapter 9). These are able to form hydrogen bonds with water molecules and set up monolayers on aqueous surfaces, with the hydrophobic ends oriented away from the water. In contrast, many insects appear to contain only hydrocarbons that do not form hydrogen bonds with water and therefore can not form oriented monolayers. Despite these considerations, the model proposed by Ramsay (1935) has held up well. The general picture that has developed is one in which cuticular lipids typically occur in a solid state at moderate temperatures and provide an excellent barrier to cuticular transpiration. As temperature increases (sometimes within the physiological range), the lipids melt and become more permeable, and water loss increases rapidly (Gibbs, 2002). The point at which water loss begins to increase is termed the critical temperature (T_C).

Experimental Tests of the Phase Transition Model

To rigorously test the phase transition model, one must demonstrate that surface lipids actually do melt at the critical temperature, and that melted lipids are in fact more permeable to water than solid ones. A variety of biophysical techniques have been applied to this problem (Table 6.1). These have included direct biophysical measurements of lipid properties, and associated measurements of water loss. In general, these studies have supported

Table 6.1 *Biophysical methods used to study cuticular lipids.*

Biophysical technique	Support for T_m model?	Reference
Capillary melting point	Yes	Beament (1945)
Electron diffraction	Mixed	Holdgate and Seal (1956)
Surface film compressibility	Mixed	Lockey (1976)
Electron paramagnetic spectroscopy	Yes	Toolson *et al*. (1979)
Differential scanning calorimetry	Mixed	Machin and Lampert (1990)
Infrared spectroscopy	Yes	Rourke and Gibbs (1999)

the phase transition model, at least to the extent that some change in lipid physical properties occurs close to an observed change in water loss (Gibbs, 2002).

One limitation of several studies listed in Table 6.1 is that the surface lipids must be extracted from the animal for analysis. It is possible that extraction will affect lipid properties by removing lipid–cuticle interactions, but direct comparison of lipid melting *in situ* and in lipid extracts suggests that this is not a major problem (Gibbs and Crowe, 1991; A. G. Gibbs, unpublished observations). A more important concern is regional variation in lipid composition and physical properties. For example, lipids on the forewings of the grasshopper, *Melanoplus sanguinipes*, melt at ~65°C, whereas those on the body and hind wings melt at ~45°C (Gibbs and Crowe, 1991). This type of variation makes attempts to correlate bulk lipid properties with cuticular permeability problematic. In addition, transitions in rates of water loss have been observed in animals whose surface lipids have been removed, suggesting that other components of the cuticle can affect transpiration (Yoder *et al*., 2005a).

Conversely, studies that investigate cuticular lipids *in situ* face difficulties if what they measure includes a signal from the underlying cuticle. For example, are changes in electron diffraction patterns (Holdgate and Seal, 1956) caused by lipid melting or thermal disruption of the underlying cuticle? Differential scanning calorimetry (DSC, Machin and Lampert, 1990) definitely generates a mixed signal of heat absorption by lipids and cuticle. An interesting recent application of calorimetry has been to study the physical properties of beeswax (Buchwald *et al*., 2006, 2008). Although beeswax does not have a waterproofing function, it has an important role in maintaining the structural integrity of honeycombs. In these studies, DSC provided detailed information on biophysical changes related to temperature. The ability to examine small samples (milligrams) has improved tremendously since Machin and Lampert's (1990) work, and further DSC studies of cuticular lipids are warranted.

Each of the studies mentioned so far is fundamentally correlative, with lipid phase behavior and water-loss rates being made with different animals. Lipid composition and physical properties can vary substantially within species, so a close correlation between these parameters is not necessarily expected. One possible explanation is a "file drawer" problem of the type alluded to by Lighton (1998); results that conflict with the dominant

paradigm may be dismissed as resulting from experimental errors. Rourke and Gibbs (1999) instead used inter-individual variation in surface lipids of *M. sanguinipes* as an experimental variable to test the phase behavior model. Lipid melting points (T_m) varied by ~15°C among animals, and were highly correlated with T_C. In a model membrane system, T_m and T_C were again strongly correlated (Rourke and Gibbs, 1999).

Lipid composition and physical properties: size doesn't matter (much)

The majority of publications on cuticular lipids involve analyses of lipid composition. Which compounds are present, and what is their function? Correlations between lipid composition and water loss have provided indirect tests of the phase transition hypothesis, under the assumption that changes in lipid composition predictably affect lipid properties. In this section, we summarize available information on how specific structural changes affect the physical properties of pure surface lipids, as well as how different lipids interact with each other.

We know the most about cuticular hydrocarbons, because they are abundant and because it is relatively easy to isolate and identify them. They are also the most hydrophobic lipid components, and so should provide the best barrier to water loss. *n*-Alkanes isolated from insect cuticles typically have chain lengths of 20–40 carbons. These can be modified by insertion of *cis* double bonds, or addition of one or more methyl groups. Relatively polar surface lipids include alcohols, aldehydes, ketones and wax esters (see Chapter 9). Given this diversity, is it possible to predict lipid phase behavior (and, by extension, waterproofing characteristics) from composition alone? If so, a large body of literature would become instantly interpretable in the context of water balance. Unfortunately, this is not the case.

Hadley (1977, 1978) was the first to try to relate variation in lipid composition to cuticular permeability via the link of lipid phase behavior. He used the relative mobility of hydrocarbons by gas chromatography (i.e. equivalent chain length) to predict the relative physical properties of HCs *in situ*. The basic assumption was that melting points (the physiologically relevant property) and boiling points (approximately measured by gas chromatography (GC) retention time) would be highly correlated, and many subsequent authors have made the same assumption. As an example of this approach, a (*Z*)-9-tricosene molecule has a GC retention time like that of an *n*-alkane of chain length 22.3, and a methyltricosane molecule has an equivalent chain length of ~23.7. Under this assumption, the melting point of tricosene would be ~45°C, between those of *n*-docosane (44°C) and *n*-tricosane (46.5°C) (Maroncelli *et al*., 1982; Gibbs and Pomonis, 1995), and methyltricosanes would melt at ~48°C. Instead, (*Z*)-9-tricosene melts at ~0°C, and methylalkanes melt 10–30°C below *n*-alkanes with the same chain length, depending on the location of the methyl branch (Gibbs and Pomonis, 1995).

The previous examples illustrate an important point regarding the effects of HC chain modification on melting points: not all HC structural changes are equivalent. In particular, chain length is the *least* important factor affecting T_m. *n*-Alkanes have high melting points because they pack well together in a crystalline state (Maroncelli *et al*., 1982). Lengthening

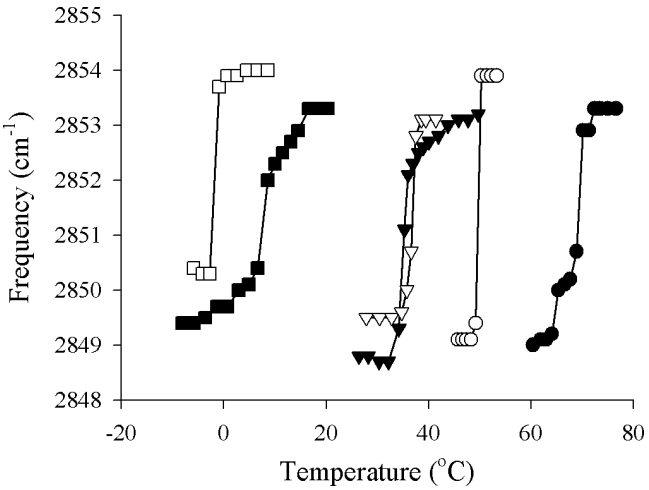

Figure 6.3 Effects of hydrocarbon chain modifications on melting points of similar-sized cuticular lipids. When lipids melt, the absorption frequency of C-H symmetric stretching vibrations increases from ~2849 cm^{-1} to ~2854 cm^{-1}. From right to left, compounds are (chemical change relative to *n*-alkane, molecular mass in daltons): filled circles, *n*-dotriacontane (no change, MM = 450); open circles, palmitoic acid myristyl ester (wax ester, 452); filled triangles, 13-methylhentriacontane (methylbranched alkane, 450); open triangles, (*Z*)-13-tritriacontene (double bond, 462); filled squares, 9,13-dimethylhentriacontane (2 methyl branches, 464); open squares, oleic acid oleyl ester (2 double bonds and an ester link, 532). Data from Gibbs and Pomonis (1995) and Patel *et al*. (2001).

of an alkane chain increases T_m by ~2°C. Insertion of a *cis* double bond disrupts crystalline structure, so that melting can occur nearly 50°C lower (see above; Gibbs and Pomonis, 1995). Other chain modifications (methyl branching, insertion of an ester linkage) also disrupt lipid packing and lower T_m.

Figure 6.3 illustrates how structural differences greatly outweigh the effects of molecular size on melting point. The lowest molecular weight compound shown, *n*-dotriacontane (MW = 450), has the highest melting point (~69°C), and the highest molecular weight compound, oleyl acid oleic ester (MW = 532), melts below 0°C. In *M. sanguinipes*, increased melting point is correlated with decreased methylbranching, despite the fact that methylalkanes in this species have longer chain lengths (Gibbs *et al*., 1991; Gibbs and Mousseau, 1994). More extensive disruptions of HC chain structure have even greater effects. The compounds considered in Figure 6.3 are essentially linear; *M. sanguinipes* synthesizes "T-shaped" compounds (wax esters of secondary alcohols; Blomquist *et al*., 1972). Despite having molecular weights averaging >600, they melt at 5–10°C (Patel *et al*., 2001). Populations with greater proportions of wax esters also have lower T_m values (A.G. Gibbs, unpublished observations). In summary, molecular mass is generally well correlated with GC retention time, but not with T_m.

Of course, insects contain mixtures of many different HCs and other hydrophobic compounds, and it is their interactions that determine the overall physical properties of the

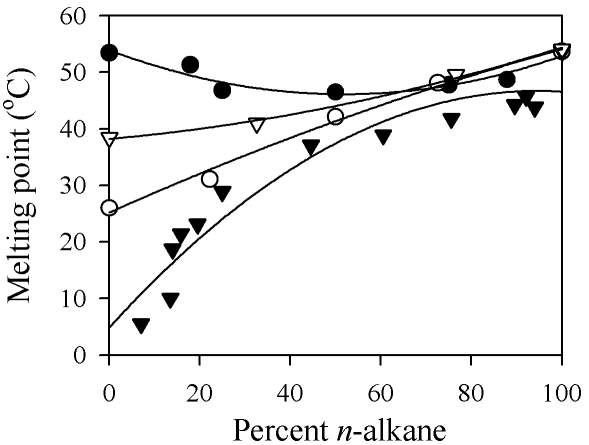

Figure 6.4 Melting points of binary lipid mixtures, each containing an *n*-alkane component, as indicated by FTIR spectroscopy. Filled circles, *n*-pentacosane and myristic acid stearyl ester. These compounds melt individually at ~53.5°C, but mixtures have lower melting points. Open circles, *n*-pentacosane and 13-methylpentacosane. The methyl branch of the latter compound reduces its melting point, but mixtures melt at intermediate temperatures equivalent to the weighted average of the components' T_m values. Filled triangles, *n*-tricosane and (*Z*)-9-tricosene. Melting points of mixtures appear higher than predicted, but this may reflect phase separation between the component lipids (Small, 1986). Open triangles, *n*-pentacosane and dodecyl acid myristoyl ester. Data from Gibbs (1995) and Patel *et al*. (2001).

surface lipids. Figure 6.4 depicts T_m values for binary mixtures of *n*-alkanes with other types of cuticular lipids. Mixtures of two different alkanes (either two straight chains or a straight-chain and a branched compound) have melting points that approximate those of the weighted average of the components, with melting occurring over a relatively wide range of temperatures (Bonsor and Bloor, 1977; Gibbs, 1995). 50:50 Mixtures of wax esters with *n*-alkanes melt up to 10°C below the expected temperature, consistent with disruption of lipid packing by the ester link (Patel *et al*., 2001).

Mixtures of alkenes with alkanes present a more complicated picture. Based on FTIR alone, mixtures appear to melt at higher than predicted temperatures (Gibbs, 1995), but other techniques reveal a much more complicated picture (Small, 1986). Alkenes found on insects generally melt at lower temperatures than the alkanes present (Gibbs and Pomonis, 1995). Saturated and unsaturated HCs crystallize separately below the alkene's melting point, then the alkene melts at its respective T_m (Small, 1986). As temperature increases further, the alkane crystals begin melting into the fluid phase, leaving a state of phase separation until the alkane is completely melted. The implications of this state for cuticular transpiration, chemical communication, defense, etc. are not understood. Given the fact that alkenes and alkanes are found on so many insects, and the communication function frequently exhibited by unsaturated HCs (Howard, 1993), further investigation of their interactions is certainly warranted.

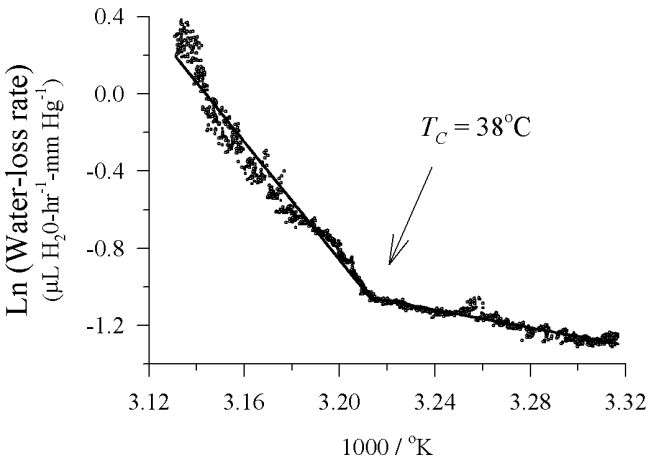

Figure 6.5 Arrhenius plot of water loss from a live grasshopper, *Melanoplus sanguinipes*. Cuticular lipids extracted from this individual melted at ~42°C. Data from Rourke and Gibbs (1999).

Theoretical consideration of the transition phenomenon

The physical–chemical theory behind cuticular permeability has been considered on several occasions (Toolson, 1978, 1980; Gelman *et al*., 1988; Noble-Nesbitt, 1991; Gelman and Machin, 1994; Yoder *et al*., 2005b). One topic of continued discussion is how T_C is determined. Critical temperature has often been estimated by visual inspection of plots of water-loss rate vs. temperature. This approach is somewhat subjective, and the apparent T_C may depend upon the range of experimental temperatures. Because water loss rates increase in a somewhat exponential fashion with temperature, larger experimental temperature ranges compress the *Y*-axis at low temperatures, leading to the impression of higher T_C values. Plotting water rates on a logarithmic scale reduces this problem to some extent, but estimating T_C remains subjective. To allow a more objective measure, T_C has been estimated as the break point on biphasic Arrhenius plots (Holdgate and Seal, 1956; Machin and Lampert, 1989; Rourke and Gibbs, 1999). In many cases, apparent critical temperatures disappear when Arrhenius plots are used (Yoder, 2005b; Yoder and Tank, 2006).

Arrhenius plots are familiar to us from basic chemistry courses. The theory was derived in the late-nineteenth century for simple chemical reactions. It has since been applied to more complicated situations, from enzymatic reactions to entire ecosystems. Figure 6.5 shows an Arrhenius plot of ln(water loss rate) versus 1/(absolute temperature) for a grasshopper, *Melanoplus sanguinipes*. At ~38°C, water-loss rates began to increase more rapidly with inverse temperature (the melting point for surface lipids extracted from this animal was ~42°C). Not all insects exhibit such a transition, at least not within the range of experimental temperatures, even when such transitions appear evident on linear–linear plots (Gibbs, 1998, Yoder *et al*., 2005b; Yoder and Tank, 2006).

The general formula for Arrhenius relationships is

$$\text{Rate} = A * \exp(-\Delta G‡/RT) = A * \exp(-(\Delta H‡ - T\Delta S‡)/RT) \quad (6.1)$$

or

$$\text{In(Rate)} = \text{In}(A) - \Delta G‡/RT = \text{In}(A) - \Delta H‡/RT + \Delta S‡/RT \quad (6.2)$$

where R is the gas constant; $\Delta G^‡$ is the *apparent* free energy of activation (i.e. the difference in Gibbs free energy between the transition and ground states of the reaction) and A is a reaction-specific factor that takes into account steric factors (i.e. the likelihood that the reactants will be in the correct orientation to react successfully) and other variables, including properties of the medium in which the reaction occurs. $\Delta H^‡$ and $\Delta S^‡$ are, respectively, the apparent enthalpy and entropy of activation. An important point is that A, $\Delta G^‡$, $\Delta H^‡$ and $\Delta S^‡$ may all depend upon temperature, even in very simple chemical reactions (Berry *et al*., 1980).

For a simple reaction, the slope of an Arrhenius plot is proportional to $\Delta G^‡$, with a steeper slope implying a higher energetic barrier to overcome. Yoder *et al*. (2005b) have recently challenged this common interpretation when applied to cuticular water loss. The problem is the upward inflection at high temperatures, with the higher slope being indicative of a higher energetic barrier to water loss. Yoder *et al*. (2005b) argue that, as cuticular waxes melt at the T_C and become more permeable, the energetic barrier to diffusion can not possibly become higher. Their point is well taken, and we discuss below some factors that may allow a better theoretical understanding of cuticular permeability. Our discussion is largely based on theoretical reviews by Silvius and McElhaney (1981) and Klein (1982), developed in the context of membrane transport processes. These are, as theoretical works go, relatively accessible to experimental physiologists, and we recommend them to mathematically oriented readers.

Transpiration through the cuticle involves more than just the single step of diffusion through the epicuticular lipid layer. Molecules of water must leave the tissues adjacent to the cuticle, diffuse through the cuticle itself, enter the lipid layer, diffuse across the lipids, and enter the gas phase outside the animal. Each step is likely to be affected by temperature to a different extent. Lipid composition and physical properties can also differ from one region of the cuticle to the next, so that the biophysical details of cuticular transpiration may not be homogeneous across the entire animal. Thus, transpiration at the organismal level involves multiple steps, and parallel routes for water flux.

Consider first a simple two-step transport reaction. It can be demonstrated theoretically (Klein, 1982) that the step with the higher value of $\Delta G^‡$ will be the slow step at low temperatures, and the low $\Delta G^‡$ step will be rate-limiting at higher temperatures, leading to a downwardly concave Arrhenius plot (note that Arrhenius plots for cuticular transpiration are concave upward). A similarly shaped curve will be obtained if the apparent activation energy decreases at high temperature, for example if a cell membrane undergoes a melting phase transition that decreases the energy barrier to activity of a membrane enzyme,

but does not change the rate-limiting step. Thus, Klein (1982) provides the mathematical underpinnings for the argument by Yoder *et al*. (2005b) that activation energy cannot increase with temperature. This is a relatively familiar example in which changes in lipid phase behavior can result in non-linear Arrhenius behavior, but several other scenarios can result in similar behavior, or even upward concavity. I will discuss the latter here, as these are potentially relevant to cuticular transpiration.

Partitioning of transport between different lipid phases. Klein (1982) considers the case of a transport reaction, in which the transporter preferentially exists in either the fluid or gel region of a membrane. An important conclusion is that, even if the apparent activation energy is the same in both phases, non-linear Arrhenius behavior can be observed. In fact, the Arrhenius curve is triphasic, when a broad enough range of temperatures is considered. In addition, depending on the partitioning of the transporter, neither of the two break points need correspond to the actual melting point of the membrane. Effectively, the *A* term in Equation (6.1) changes with phase state. A potential mechanistic explanation might be that reduced membrane viscosity allows the transporter to turn over more rapidly when it enters the fluid-phase membrane, although the activation energy does not change. How might this relate to cuticular transpiration? Water moves through membranes via transient defects in lipid packing (Carruthers and Melchior, 1983; Deamer and Bramhall, 1986). These defects increase as lipids melt, and indeed what FTIR spectroscopy actually measures is lipid defects (*trans-cis* isomerizations of hydrocarbon chains). The more defects available, the more water molecules will partition into the lipid layer, and the better the chance that molecules will diffuse through the lipid layer. Melting of cuticular lipids therefore may effectively increase the solubility of water in the lipid layer, thereby increasing the overall flux. In analogy to the models derived by Klein (1982), more defects ("transporters") are available in fluid lipids, and so water flux will increase more rapidly than if one considers the permeability of just solid or melted lipids alone.

Multiple routes for transpiration. The insect cuticle is a complex structure. Different types of cuticular lipids may be deposited on different regions of the body, resulting in different physical properties (Gibbs and Crowe, 1991; Young *et al*., 2000; see also discussion in Chapter 20). At even finer scales, surface lipids may form non-homogeneous mixtures of melted and solid regions (Small, 1986; Gibbs, 2002). Additionally, the amount of lipid deposited may vary, and underlying cuticular layers may have differing permeabilities (Hadley and Quinlan, 1987). Each route may differ in its temperature sensitivity, and this may cause non-linear Arrhenius behavior. In particular, at low temperatures, cuticular lipids may provide such a strong barrier to water loss that other water-loss routes dominate. If transpiration through lipid barriers has a higher apparent activation energy ($\Delta G^{\ddagger}$) than flux through other routes, trans-lipid fluxes will increase more rapidly with temperature, and an upward inflection of an Arrhenius plot will be observed. This can happen whether or not the lipids actually melt.

Although originally derived for membrane transport phenomena, the mechanisms outlined above can also provide potential explanations for non-linear, upwardly-concave Arrhenius plots for passive diffusion (Silvius and McElhaney, 1981; Klein, 1982). Other

biophysical explanations can also explain these findings (e.g. a negative heat capacity of activation). The take-home message is that Arrhenius plots alone provide little information regarding underlying physical mechanisms of reactions or processes. Sharp break points on Arrhenius plots may not occur, even if there is a sharp lipid phase transition (Silvius and McElhaney, 1981). Break points can occur without a change in rate-limiting step, and changes in rate-limiting steps can occur without break points (Klein, 1982). Finally, $\Delta G^{\ddagger}$ is affected by temperature, even in simple reactions (Berry *et al*., 1980). The temperature dependence of $\Delta G^{\ddagger}$ is usually small, but differs depending on the reaction mechanism. Movement of water through the insect cuticle is a complex process, and there is clearly much to be learned from further experimental and theoretical work.

Are cuticular lipids the entire story? Melanization and water loss

Not all studies find a relationship between water loss and surface lipid amounts, composition or melting point, including numerous studies using *Drosophila*. A comparative study found that the longest chain lengths and highest melting points occurred in species from cool boreal forests, species which lost water the fastest (Gibbs *et al*., 2003). Within species, Indian populations of *D. immigrans* differ in water-loss rate, but not in surface lipid amounts (Parkash *et al*., 2008c). In a laboratory selection experiment, populations selected for desiccation resistance lost water ~50% less rapidly than unselected controls, but the two groups exhibited minor differences in lipid composition and T_m (Gibbs *et al*., 1997). Thermal acclimation of the desert fly, *D. mojavensis*, results in substantial changes in HC composition, but relatively little change in water-loss rates (Gibbs *et al*., 1998). It must be noted that not all studies result in negative findings (e.g. Toolson, 1982; Toolson and Kuper-Simbrón, 1989).

Why do so many *Drosophila* studies report no relationship between surface waxes and water loss? There are several possibilities. One is that *Drosophila* have relatively high respiratory water loss rates (Lehmann *et al*. 2000; Lehmann, 2001), so that cuticular water loss is relatively unimportant. Inter-specific correlations between water loss and metabolic rate are consistent with this idea (Gibbs *et al*., 2003). Cuticular water loss accounts for >80% of total water loss in most insects studied to date (Gibbs and Quinlan, 2006). *Drosophila* can regulate spiracular tone to some extent (Lehmann, 2001; Gibbs *et al*., 2003), but whether they normally do so is unknown. *Drosophila* may also be constrained in the degree to which they can evolve surface lipids that restrict water loss better. Cuticular hydrocarbons in *Drosophila* are used in chemical communication as species- and sex-recognition pheromones (Howard, 1993). Changes that conserve water but reduce a fly's mating success will not spread in a population unless water stress is an important factor affecting survival. Even in deserts, *Drosophila* usually have access to rotting plant material to replenish lost water (Breitmeyer and Markow, 1998).

Finally, it must be noted that the cuticle contains other components besides lipids. The cuticle is a complex structure, and its components vary greatly between species and

populations. With regard to water balance, melanin may be a particularly important component. Significant structural integrity of the cuticle is provided by aromatic cross-links inserted between adjoining polypeptide chains, causing progressive hardening, dehydration, and close packing (Hopkins and Kramer, 1992). Hardening and darkening of the cuticle share the same biochemical processes (Franenkel and Rudall, 1940; Pryor, 1940). Dark cuticle achieves its color largely because of the deposition of melanin granules (polymers of dopa and other tyrosine derivatives; True, 2003). Melanin is also hydrophobic and therefore may reduce the permeability of the cuticle. In this light, it is interesting to note that light-colored *yellow Drosophila* mutants lose water faster than darker wildtype flies, while dark *ebony* mutants are more desiccation resistant (Kalmus 1941; Da Cunha 1949).

A series of recent studies have examined clines in melanization and water balance in Indian populations of *Drosophila* (reviewed by Rajpurohit *et al*., 2008a). Temperature is negatively correlated with latitude and altitude on the Indian subcontinent (Parkash *et al*., 2008d; Rajpurohit *et al*., 2008b). Populations of *Drosophila melanogaster* and *D. immigrans* exhibit increased melanization in cooler regions (Parkash *et al*., 2008c,d), consistent with a need to maintain a high body temperature to allow flight and other activities. The importance of melanization for temperature regulation in drosophilids and other small insects needs to be established experimentally, however. Willmer and Unwin (1981) reported that insects the size of *Drosophila* do not attain temperatures even 1°C above ambient, whereas large (>100 mg), dark insects with poorly reflective bodies can achieve body temperatures >10°C above ambient.

An alternative explanation for clines in melanization is suggested by the fact that humidity also decreases with latitude and altitude in India (Parkash *et al*., 2008b, d). Darker populations of several *Drosophila* species have relatively low water-loss rates compared to lighter populations, despite a lack of differences in surface lipid quantities (Parkash *et al*., 2008a, c; Rajpurohit *et al*., 2008a). In contrast, populations of a light-bodied drosophilid, *Zaprionus indianus*, exhibit the same latitudinal cline in water loss, without pigmentation variation, but with an increase in HC amounts in drier areas (Figure 6.6; Parkash *et al*., 2008a). These results suggest that there can be alternative "strategies" for reducing cuticular transpiration and coping with arid environments.

Additional evidence supporting a role for melanization in water conservation comes from a study of *D. polymorpha* populations in Brazil (Brisson *et al*., 2005). Dark flies were most abundant in open, dry environments, despite the fact that these were warmer than more humid forest habitats, where light flies predominated. In this case, melanization patterns were opposite to those expected for thermal regulation, but consistent with a role in water conservation. Thus, in combination with studies by Parkash and colleagues, there is increasing evidence that melanization significantly affects water balance, at least in *Drosophila*. The fact that transitions in water loss can be observed in solvent-extracted animals (Yoder and Tank, 2006) also supports a role for non-lipid layers in determining cuticular permeability. Lipids are probably the primary barrier to cuticular transpiration, but other cuticular barriers may be important and should not be ignored.

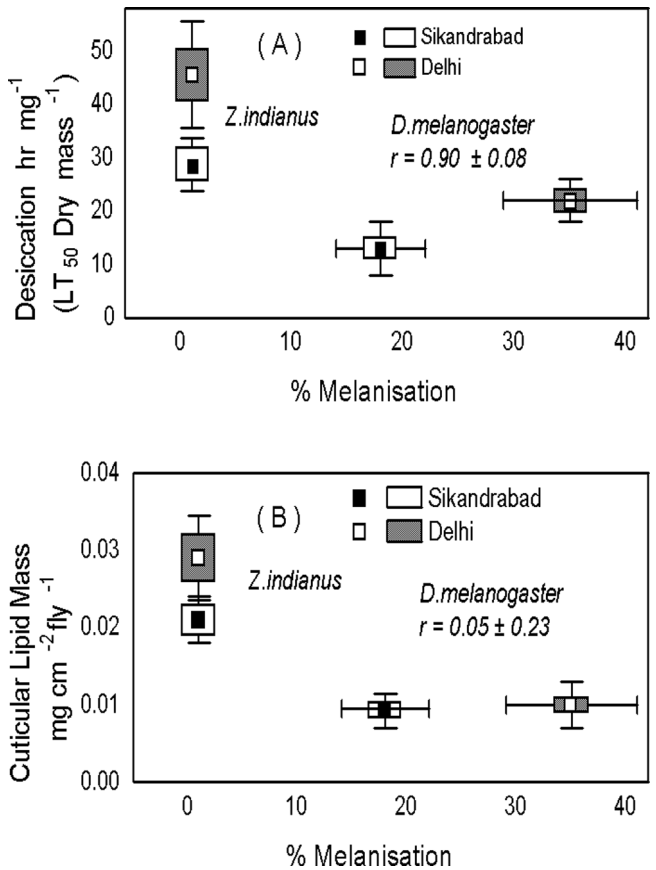

Figure 6.6 Within and between population variation in desiccation resistance and cuticular lipid quantity as a function of body melanisation in northern and southern Indian populations of *Zaprionus indianus* and *Drosophila melanogaster*. In both species, populations from drier (northern) habitats are more desiccation resistant. The desiccation-resistant population of *D. melanogaster* is melanic, whereas desiccation resistance in *Z. indianus* is correlated with higher amounts of surface lipids. From Parkash *et al*. (2008a); used with permission.

Finally, even when HC composition and cuticular transpiration are correlated, causation can not be assumed. For example, higher cuticular water-loss rates in the desert ant, *Pogonomyrmex barbatus*, are correlated with a decrease in abundance of an *n*-alkane and an increase in a methylalkane (Figure 6.2; Johnson and Gibbs, 2004). This is exactly what one would expect if lipid melting points affect cuticular permeability, but this increase is also accompanied by a change in mating status. Mated, de-alate queens that have founded colonies lose water most rapidly, but they also have undergone the physical stress of repeated mating in large aggregations, followed by soil abrasion during colony founding

(Johnson, 2000). Both HC composition and cuticular damage may contribute to higher cuticular water loss in this species (Johnson and Gibbs, 2004).

Summary

The function of epicuticular lipids in reducing insect water loss has been recognized for almost a century. Despite this early recognition, our understanding of the process by which water moves through the cuticle is limited. Transpiration rates are strongly affected by temperature, probably via temperature's effects on lipid phase behavior. Solid-phase lipids provide a tighter barrier to transpiration than melted ones, but we know relatively little about how different compounds interact to determine cuticular permeability. Many biophysical techniques have achieved orders of magnitude improvements in sensitivity and precision since they were last applied to insects, and some (e.g. NMR, atomic force microscopy) have never been applied. More speculatively, researchers in nanotechnology have developed the ability to create and study objects and materials smaller than the smallest insects; some of these tools should be applicable to understanding cuticular structure and function. Finally, several recent studies have highlighted the potential importance of other cuticular properties, specifically melanization, in conserving water. Although surface lipids are clearly important in determining cuticular transpiration, the cuticle is a complex structure, and lipids are only part of the picture.

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