Molecular and Physiological Responses of Hematophagous Arthropods to Dehydration

Dissertation

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By

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Abstract

Dehydration tolerance in terrestrial arthropods is defined by two factors: an individual's ability to maintain water balance and capability to respond to the stress generated during fluctuations in the water content. To maintain water balance, an arthropod has to balance water loss and water gain. This is accomplished by reducing water lost through cuticular and respiratory route, improving water re-absorption by the alimentary canal or by increasing water uptake by drinking or absorbing water vapor. Water stress is alleviated by increasing the internal concentrations of protective sugars and polyols and up-regulating stress-related proteins that repair damaged proteins, reduce oxidative stress and maintain cellular integrity. In this thesis, select underlying molecular and physiological changes during dehydration in blood feeding arthropods were examined.

Fully hydrated *Aedes aegypti*, *Anopheles gambiae* and *Culex pipiens* females contained nearly the same amount of water (66-68%), but water loss rates differed among the species, with *A. aegypti* having the lowest water loss rate (2.6%/h), followed by *C.pipiens* (3.3%/h), and *A. gambiae* (5.1%/h). In all three species water could be replaced only by drinking water (or blood). Diapause in *C. pipiens* improved the ability of females to resist dehydration. Multiple dehydration bouts reduce the nutritional reserves of mosquitoes, likely due to the cost of responding to

dehydration stress, leading to reduced survival and reduced egg production.

Dehydration elicited expression of *hsp70*, and *hsp90* was constitutively expressed in *A. gambiae*, *A. aegypti*, and *C. pipiens*. Injection of dsRNA to knock down expression of *hsp70* and *hsp90* in *A. aegypti* did not alter water content or water loss rates, but the dehydration tolerance was lower. Instead of surviving a 36% water loss, females were able to survive only a 28% water loss in response to RNAi directed against *hsp70* and a 26% water loss when RNAi was directed against *hsp90*.

The bed bug, *Cimex lectularius*, and the seabird tick, *Ixodes uriae*, are much more resistant to dehydration than mosquitoes. Both arthropods have low water loss rates and they further reduce water loss by forming aggregations. Bed bugs are incapable of absorbing water vapor from the air and rely solely on blood for liquid water. In contrast, the seabird tick absorbs water from the atmosphere but cannot drink free water. Bed bug water loss rates increase in response to alarm pheromone components, (E)-2-hexenal and (E)-2-octenal, presumably due to the increase in bed bug activity elicited by the alarm pheromones. When these chemical were applied in combination with insecticidal desiccant dust, the effectiveness of this control method increased by nearly 50%.

Overall, these experiments define the water balance characteristics of mosquitoes, the common bed bug, and seabird ticks. Establishing the water balance profiles of these arthropod vectors is a critical aspect for determining their possible distribution and impact on public health. Dedication

Dedicated to my mom, Stacey, for her love and support.

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Field of study

Major field: Entomology

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Chapter 1: Introduction

Overview

Water balance of terrestrial arthropods represents a very diverse field with significant differences among closely related species. For the Introduction, two separate reviews are provided on the water balance of terrestrial arthropods. The first is a section on the water balance requirements of blood feeding arthropods, and provides information on the response of arthropod vectors to the water stress of blood feeding and how individuals maintain water balance between bloodmeals. The second section provides information on the dehydration resistance of insects during periods of dormancy. Discussion of mechanisms utilized to prevent water loss, increase water pool and prevent/respond to dehydration-induced stress are included in this section. Additionally, water balance changes during dormancy are critical since many hematophagous arthropod enter periods of aestivation, diapause and quiescence when environmental conditions become adverse or when host are not present. Overall, these two sections of the Introduction provide a comprehensive review of water balance, particularly for blood-feeding arthropods.

Meeting the challenges of on-host and off-host water balance in blood feeding arthropods

Abstract

Blood feeding has developed independently numerous times in terrestrial arthropods. Several previous reviews have focused on the water balance requirements of arthropods during blood feeding or during the off-host phase between bloodmeals. In this review, we provide a comprehensive description of the water balance requirements of blood-feeding arthropods, particularly focusing on changes in dehydration tolerance during the transition from the unfed, off-host state to the fully engorged state after blood feeding. First, we discuss basic water balance characteristics that apply to all terrestrial arthropods and indicate how these characteristics relate to the off-host physiology of hematophagous arthropods, their distribution, and impact on public health. Second, we discuss changes that occur during blood feeding to prevent overhydration and to condense the bloodmeal. Third, we discuss how the developmental transition to the next stage or a return to the unfed state alters water balance. To reduce the amount of water from the bloodmeal, most insects begin diuresis during or immediately after blood feeding and increase their rate of cuticular water loss. Following bloodmeal digestion, either reproduction or stage advancement occurs, leading to individuals that are usually more resistant to dehydration (larger size or deposition of additional cuticular hydrocarbons generated from the bloodmeal). Finally, the blood feeder will complete its transition back to a dehydration-resistant off-host state and prepare to once again initiate host seeking.

Diverse arthropods that feed on copious amounts of blood encounter similar stresses, particularly the sudden influx of water, and similar physiological mechanisms are engaged to both counter the influx of water from the bloodmeal and to return to the nonfeeding off-host state.

Introduction

A blood-feeding lifestyle imposes an interesting set of water balance challenges for arthropods. Though the blood meal is a rich nutrient source, the huge amount of water it contains demands special mechanisms for quick extraction and expulsion of excess water. And, contrasting mechanisms for conserving water are required during the long periods of off-host life. The life of blood feeder is a continual cycle of overhydration during blood feeding followed by the suppression of dehydration while off the host (Wharton, 1985; Hadley, 1994; Ribeiro, 1996). This implies that throughout its life a blood feeder goes through multiple cycles switching from water conservation to getting rid of unwanted water.

Many water balance attributes of the off-host phase of the blood feeder's life cycle are common to all terrestrial arthropods, and excellent earlier reviews by Edney, (1977), Wharton (1985) and Hadley (1994) provide good summaries of the basic principles of water balance that are germane to the off-host phase of the life cycle. Several excellent reviews also discuss various aspects of the rapid processing of water removal during blood feeding: transport mechanisms in the gut and Malpighian tubules (Beyenbach 2003; Lehane, 2005), and the hormonal regulation of diuresis (Coast et al., 2002).

In this review we provide an update of off-host water balance, the changes that occur during blood feeding, and the responses observed during reversion to the non-feeding state. This review focuses only on the hematophagous stages of bloodfeeding arthropods (e.g. adult mosquitoes, both nymphal and adult stages of ticks), not the immature stages that do not feed on blood (e.g. mosquito larvae). We suggest that the off-host water balance traits impact the distribution of hematophagous arthropods and offer predictive value for delineating future population expansion.

Blood-feeding arthropods

Blood feeding has evolved in diverse groups of arthropods (Table 1). The vast taxonomic distances among blood feeders and the diverse methods they employ to obtain a blood meal suggest that blood feeding evolved numerous times within the Arthropoda (Waage, 1979; Klowden, 1996). More than 14,000 species representing 400 genera utilize blood from vertebrate hosts (Graça-Souza et al., 2005). Blood feeding likely developed by three distinct routes (Klowden, 1996). In the first scenario arthropods living in close association with vertebrates feed on exfoliated skin or other host by-products (Kim, 1985; Lehane, 1991; Klowden, 1996). As the association progressed these arthropods began to utilize the more nutrient-rich blood due to the development of chewing or piercing mouthparts capable of penetrating the vertebrate epidermis. Once blood was regularly utilized, groups feeding on protein-

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rich blood were favored due to increased egg production (Lehane, 1991). This difference is apparent for lice; blood-feeding Anoplura produce significantly more eggs than skin-feeding Mallphaga (Marshall, 1981). The alternative possibility is that arthropods capable of piercing plant material made a switch to blood feeding to obtain a more protein-rich meal (Waage, 1977). The common example of this is the vampire, *Calpe eustrigata*, a Noctuidae that has a modified proboscis to penetrate fruit rinds, but also obtains blood from vertebrates. Third, entomophagous insects have mouthparts capable of penetrating the cuticle of arthropods (Stys and Daniel, 1957; Lehane, 1991). If continual interactions occur between entomophagous insects and vertebrates, blood ingestion may occur. The hemipteran, *Lyctocoris campestris*, resides in bird nests and primarily feeds on other insects but will also utilize blood from nearby birds (Stys and Daniel, 1957).

It is important to note that blood feeding is not a trivial task. Rather, many inhibitory factors need to be overcome before an arthropod vector can utilize blood. The first factor is locating a host. To accomplish this, the arthropod either actively quests for a host or employs an ambush strategy, waiting in a particular area for a host to appear (Bowen, 1991; Bowen et al., 1998; Klowden, 1996). In both strategies, host-based chemical cues are utilized. Those actively searching move toward the host prompted by chemical cues such as host volatiles and carbon dioxide as well as visual cues in some cases (Gilles, 1980; Lehane, 2005), and those that wait passively usually reside in areas frequented by their hosts, often indicated by the presence of feces, urine or other chemical cues (Klowden, 1996; Benoit et al., 2009). Host seeking is terminated after blood feeding by abdominal distension and hormones released by the

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ovaries during egg maturation (Klowden, 1990; Brown et al., 1994). Once a host has been located, even with the proper mouthparts to penetrate the vertebrate integument, the arthropod must suppress the host immune response, increase vasodilation, prevent the accumulation of toxic compounds, and suppress platelet aggregation and general coagulation in order to successfully blood feed (Ribeiro, 1995; Ribeiro, 1996; Stark and James, 1996; Ribeiro and Francischetti, 2003; Graça-Souza et al., 2005). For insects that feed only on blood, such as bed bugs and tsetse flies, microbial symbionts are needed to synthesize required micronutrients, such as B vitamins and other factors needed for growth and development (Romoser, 1996; Ribeiro, 1996; Beard et al., 2002; Akman et al., 2002; Aksoy and Rio, 2005). Additionally, a huge blood meal represents an enormous mass change that not only makes individuals vulnerable to predation, but represents a significant osmotic stress due to the amount of water and ions within the bloodmeal (Adams, 1999; Beyenbach, 2003). Thus, the utilization of vertebrate blood is a significant feat, from locating the host to dealing with stress induced by the blood meal, but the substantial nutrients available for development and egg production make blood feeding highly rewarding (Beyenbach and Petzel, 1987; Lehane, 2005).

Basic concepts of water balance

The maintenance of water balance can be expressed using the following equation (eq. 1),

 $m = m_{\rm S} - m_{\rm T} \tag{1}$

where *m* is water mass, $m_{\rm S}$ represents water uptake, and $m_{\rm T}$ is the net transpiration rate (Wharton, 1985). For an insect to survive, water mass has to be maintained over extended periods to remain active and reproduce.

In relation to water balance, there are two distinct types of blood feeding. The first includes adult feeding patterns noted in most dipterans, lice, and bugs. Individuals become engorged with blood and return to their normal hydration state within a distinct period, usually after diuresis and subsequent egg production (Fig. 1a). Thus, water content and insect size are similar before and after blood feeding. Ixodid ticks represent an exception since the adults die after mating and egg laying, thus failing to return to the pre-feeding state (Sonenshine, 1991). The second situation is when blood feeding prompts advancement to the next stage (Fig. 1b). In this case, blood feeding triggers a molt rather than egg production, thus requiring a completely different set of water balance parameters. Additionally, once the next developmental stage is attained, individuals are more resistant to dehydration due to their larger size (Benoit, 2009).

Off-host water balance maintenance

Between bloodmeals, the challenge is to maintain sufficient body water. In this regard, blood-feeding arthropods can be classified into two distinct groups: those that have the ability to increase their water pool when off-host and those that are unable to do so. Arthropod vectors that fail to drink free water include many species of bugs, fleas and some ticks (Hadley, 1994; Thiemann et al., 2003; Benoit et al., 2007a,b). The group that uptakes water while off-host can be further subdivided into those that drink free water, e.g. mosquitoes, and those that can absorb water directly from the air, e.g. ticks. Vectors with the ability to increase their water pool when offhost have a tendency to be less resistant to dehydration when compared those that rely solely on water from their bloodmeal. In this section, we discuss how blood-feeding arthropods, when off-host, reduce water loss, increase their water pool, and prevent dehydration stress.

Reduction of water loss

To retain water more efficiently, arthropods utilize mechanisms to reduce water loss through the cuticle and during respiration, as well as increase re-absorption in the alimentary canal. Water lost through the cuticle accounts for a significant portion of the water lost by insects (Hadley, 1994; Chown and Nicolson, 2004). Commonly, individuals that frequent moist habitats have highly permeable cuticles and those that reside in more xeric regions have cuticles more resistant to water flux (Edney, 1977; Hadley, 1994; Gibbs, 1998; Bradley et al., 1999; Gibbs and Matzkin, 2001). Increases in scleritization of the cuticle increase the density of the procuticle, thus improving water retention (Benoit et al., 2005), but the cuticular lipids provide the most significant water barrier. Cuticular lipids located on the outer surface of the epicuticle, particularly the wax layer play a major role in reducing water loss (Blomquist et al., 1987; Hadley, 1981; Lockey, 1988; de Renobales et al., 1991; Hadley, 1994; Gibbs, 1998; 2002b). The composition of epicuticular lipids varies significantly between different arthropods, but the dominant constituents of most terrestrial arthropods are hydrocarbons and closely-related products (Blomquist et al., 1987; Hadley, 1994; Gibbs, 1998; 2002b), and disruption of these lipids increases water loss rates (Noble-Nesbitt 1991, Hadley 1994). Both the quantity and quality of epicutcular lipids can impact water loss rate. Increases in the amount of cuticular hydrocarbons result in lower cuticular water loss rates (Hood and Tschinkel, 1990; Yoder and Denlinger, 1991; Hadley, 1994; Benoit and Denlinger, 2007; Benoit et al., 2008a), and shifts in hydrocarbon composition toward more long-chained, saturated lipids with few methyl side chains result in a more effective water barrier (Hadley, 1994; Gibbs, 1998).

Along with water loss through the cuticle, respiration represents an important route of water loss, accounting for 5-20% of the total water loss for most insects' (Hadley, 1994; Chown, 2002). Insects residing in arid regions usually lose a higher proportion of water through respiration, up to 70%, mainly a consequence of having a highly water-proofed cuticle (Hadley, 1994; Chown, 2002). During respiration, water is lost rapidly when the spiracles are open due to the steep humidity gradient between the tracheal system and the environment (Hadley, 1994). The most prominent mechanism to reduce respiratory water loss is the most simple, closing the spiracles. By closing or even partially blocking the spiracles, water loss, particularly at low relative humidities, is reduced (Bursell, 1957; Hadley, 1994; Chown, 2002). Discontinuous gas exchange (DSC) can also reduce water loss through the spiracles by limiting gaseous diffusion to short periods when carbon dioxide accumulates at a high level (Lighton et al., 1993; Hadley, 1994). Recent research has questioned the ability of DSC to reduce water loss, indicating that more studies are needed to determine exactly how DSC relates to water loss suppression (Sláma, 1999; Chown, 2002), but clearly a lack of spiracle control results in higher water loss rates (Bursell, 1957; Lighton, 1996; Sláma, 1999; Chown, 2002).

The alimentary canal is responsible for the regulation of salt and water levels. The regions of the alimentary canal that regulate a majority of the fluid levels are the Malphigian tubules and the hindgut, divided into the ileum and rectum. Many studies have focused on the role of the alimentary canal on water and osmotic regulation, as reviewed by Bradley (1985) and Chown and Nicolson (2004). Briefly, water, along with organic molecules, particularly urine and ions, are absorbed from the hemolymph into the upper portion of the Malphigian tubules, and the urine is then actively concentrated in the lower portion of the tubules. The hindgut, particularly the rectum, acts as the primary site for the re-adsorption of water and select solutes. Secretion and absorption by the Malphigian tubules and hindgut reabsorption are regulated by neuropeptide hormones: diuretic hormones cause the secretion of water into the alimentary canal, thus increasing the net water loss, while anti-diuretic hormones act in the opposite direction to retain water. The major neuropeptides involved in water balance are summarized in papers by Coast et al. (2002), Riehle et al. (2002), Gäde (2004) and Coast (2006), and include calcitonin-like peptides, corticotropin-releasing factor related peptides (CRF-related), insects kinins, and cardioacceleratroy peptides, which all function as diuretic hormones, and chloride transport-stimulating hormone (CTSH) and an ion-transport process peptide (ITP)

that serve as anti-diuretics (Coast, 2006). A more comprehensive description of diuresis following blood feeding, providing detailed information on this physiological process, is provided later in the review.

Uric acid is the dominant nitrogenous waste product produced by terrestrial arthropods, but guanine and other closely-related nitrogenous products are used by ticks and spiders (Hadley, 1994; Benoit et al., 2008). Why use uric acid rather than urea or ammonia? Ammonia is toxic and soluble, thus requiring insects to quickly expel this waste product with large quantities of water. Utilization of ammonia as a metabolic end product is thus usually restricted to aquatic insects. Although urea is significantly less toxic than ammonia, it still has to be eliminated in solution. Uric acid is the least toxic of the potential waste products for insects, and due to its low solubility, excretion of a nearly dry waste product is possible. Also, uric acid can be accumulated within the body of the insect (storage excretion) due to its low toxicity, a situation that completely prevents a loss of water by defecation.

Behaviorally, blood-feeding arthropods can reduce water loss by multiple methods. Aggregation is a frequent behavior response associated with reducing water loss, particularly during dormant periods (Benoit, 2009). Formation of an aggregation increases the local relative humidity, suppressing water loss for members of the group (Yoder et al., 1993; Benoit et al. 2007a,b). For example, bed bugs *Cimex lectularius*, form aggregations in protective harborages near their host (Benoit et al., 2007a). As the group size increases, metabolic rate drops and water conservation is enhanced (Yoder et al., 1993; Benoit et al., 2007). These benefits gained by forming an aggregation are likely adaptive advantages forming the basis for the frequently observed clusters of off-host blood-feeding arthropods. Another factor that can reduce water loss is the restriction of host seeking to times of the day or to seasons when relative humidities are high, as noted for kissing bugs, mosquitoes, and ticks (Barrozo et al., 2003; Crooks et al., 2006; Kessler and Guerin, 2008); when they are not host seeking, vectors commonly reside in cool, moist habitats (Kessler and Guerin, 2008). Thus, blood-feeding arthropods susceptible to dehydration are most likely to reside in humid microenvironments, possibly in aggregations, and feed when the relative humidity is high, such as at night or after a rain storm, reducing the likelihood of dehydration. Those more xerically-adapted are likely to be found in drier habitats, but even these species are likely to aggregate to reduce dehydration (Benoit et al., 2007).

Increasing the water pool

Ingestion of water represents the main route used by terrestrial insects to replenish their water content (Hadley, 1994). Many insects simply drink free standing water to rehydrate, and this is usually regulated by hemolymph volume (Chown and Nicolson, 2004). Mosquitoes that are not blood feeding primarily increase their water pool by drinking free water or nectar or by obtaining fluid from plant tissue (Benoit et al., 2009), while bed bugs, which are capable of drinking, do not do so and only obtain water from blood (Benoit et al., 2007). Metabolism of food resources also generates water, and this water source is immediately transferred to the insect's water pool (Edney, 1977; Hadley, 1994). But, the contribution of metabolic water is rather small for most insects, and is probably significant only for species with extremely low water loss rates and for those that engage in extended flight. Quite a few arthropods, including acarines, lice and flea larvae, are able to absorb water from subsaturated air (< 99% RH; Hadley, 1994). A relatively complete list of arthropods capable of absorbing water vapor from the air was presented by Edney (1977), with later examples added by Hadley (1994). Potential sites utilized by insects for active water vapor absorption are the mouth and anus, and rarely water may be absorbed directly through the cuticle (Hadley, 1994; Bayley and Holmstrup, 2001). Oral and anal sites utilize hyperosmotic or hygroscopic secretions to absorb water (Knülle, 1984; Hadley, 1994). Ticks commonly use oral mechanisms of water vapor absorption to increase their water pool: larvae absorb water from the lowest relative humidities (80-85% RH), followed by nymphs (85-90% RH), and lastly adults (90-95%; Needham and Teel, 1986; 1991). This allows ticks to reside and then quest for a host in areas with little free water as long as the local relative humidity is high.

Reducing water stress

Heat shock proteins (Hsps) are among the most studied proteins in relation to arthropod dehydration (Tammariello et al., 1999; Bayley et al., 2001; Hayward et al., 2004; Sinclair et al., 2007; Lopez-Martinez et al., 2008; 2009). Thus far, expression of three Hsps (smHsp, Hsp70, and Hsp90) has been noted during arthropod dehydration (Hayward et al., 2004; Sinclair et al., 2007; Benoit et al., 2009), and in some cases one suite of Hsps is expressed during dehydration and a different suite during rehydration (Hayward et al., 2004; Lopez-Martinez et al., 2009). Increased Hsps act to either prevent stress damage due to unwanted biochemical interactions, by repairing damaged proteins or by prompting the disassembly and breakdown of proteins damaged during dehydration (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). Recently, we have shown that Hsps are essential for mosquitoes to attain their maximal dehydration tolerance: using RNA interference to suppress expression of *hsp70* and *hsp90*, we demonstrated a reduction of dehydration tolerance in Aedes aegypti (Benoit et al., 2009b). Late embryogenesis abundant proteins (LEAs) are also likely players; the LEAs appear to act by stabilizing protein structure as the water content within the insect declines (Kikiwada et al., 2008). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), are elevated during dehydration, presumably to reduce damage from oxygen radicals formed from desiccation-induced stress (França et al., 2007; Lopez Martinez et al., 2009). Changes in membrane and cytoskeletal proteins may be a fairly common response to dehydration (Li et al., 2009). One critical function of proteins involved with the cell membrane is to restructure the membrane to reduce water movement in to and out of the cells as the hemolymph osmolality changes. Cytoskeletal protein changes serve to stabilize the cells during the pressure and size changes caused by the osmotic stress of dehydration (Li et al. 2009). Additional proteins extremely important for regulating water levels are channel proteins, such as aquaporins (Campbell et al., 2008; Spring et al., 2009). Three families of aquaporins have been identified from invertebrates(DRIP, BIB, and PRIP families), and all of these appear to be critical for maintaining water content within cells particularly during feeding (Campbell et al., 2008; Spring et al., 2009).

Insect hemolymph osmolality ranges between 100 to 1400 mOsm kg⁻¹ and for most insects is around 400-500 mOsm kg⁻¹ (Hadley, 1994). It is important to note that increasing osmolality, even 2-3x, reduces water loss only slightly, and the net water flux out of the insect persists unless their local environment is at saturation or above their internal water activity (Willmer, 1980; Haldey, 1994; Chown and Nicolson, 2004). The alimentary canal efficiently regulates ion content and maintains osmolality in the 200-300 mOsm kg⁻¹ range for most insects that reside in mesic and xeric regions. Poor osmoregulators have osmolalities that may vary nearly 1000 mOsm kg⁻¹ (Hadley, 1994; Benoit, 2009), and such insects usually reside in moist microhabitats and are capable of tolerating high levels of water loss. Osmolality fluctuations are influenced by diverse molecules, including salts (NaCl, KCl), polyols (glycerol), sugars (trehalose), free amino acids (proline, etc.), and free fatty acids.

Many molecules that increase during dehydration have protective qualities (Goyal et al., 2005). Trehalose and glycerol are two of the most common molecules that can suppress water loss and reduce stress (Yoder et al., 2006; Watanabe, 2006). Trehalose is especially important during severe dehydration by preventing unwanted protein interactions, decreasing metabolism by altering fluid dynamics and protecting proteins and cellular membranes (Crowe et al., 1992; Suemoto et al., 2004; Goyal et al., 2005; Yoder et al.m 2006). Proline, as a free amino acid, may have similar effects (Yancey, 2005; Ignatova and Gierasch, 2006). Proline has been documented to increase during stress in a few insects (Michaud and Denlinger, 2007; Michaud et al., 2008), but future studies are needed to determine the exact function of this amino acid

during dehydration. Dehydration-induced changes have also been documented for glucose and sorbital (Hadley, 1994; Benoit, 2009).

Another factor that contributes to regulation of osmolality and water content is volume regulation and/or compartmentalization (Zachariassen and Einarson, 1993; Hadley, 1994; Zachariassen and Pedersen, 2002). For example, a significant portion of water may be lost from one water pool (i.e. the hemolymph), but water in another region of the body (e.g. salivary glands and midgut) may remain relatively constant. Typically, water in tissues is conserved at the expense of the hemolymph. This is particularly intriguing since osmolality of the hemolymph is usually higher than that within the cells during dehydration. Even though the exact mechanism for retaining water in a tissue at the expense of the hemolymph are not known it is an extremely important mechanism for retaining the integrity of biologically active tissues. Much of the stress induced by dehydration can be reduced by regulating osmolality and the water pool.

Increase in body size is another important factor for enhancing dehydration tolerance of insects. As noted in *D. melanogaster*, increased water content (from 25 to 30%) and general size, along with respiratory changes, were the key factors that increased dehydration resistance in selection experiments (Gibbs et al., 1997; Folk et al., 2001). Interestingly, these changes were not seen in cactophilic, desert-adapted *Drosophila* while they were evident in mesic species, thus raising the question whether size changes are ecologically relevant or can only be used for inter-species comparisons (Gibbs and Matzkin, 2001). Size changes have also been noted during dormancy in some insects (Hahn and Denlinger, 2007). The size of the northern

house mosquito, *C. pipiens*, increases substantially in preparation for winter (Benoit and Denlinger, 2007), but the water pool does not increase. Instead, this represents an increase in dry mass, but even so, the decrease in the surface area to volume ratio reduces water loss (Benoit and Denlinger, 2007). Increasing body size, particularly body water content, is a fairly simple mechanism for increasing dehydration resistance in insects.

Although numerous studies have focused on the response of insects to dehydration, few have assessed physiological changes resulting from multiple dehydration exposures. In *Culex pipiens*, each dehydration bout resulted in a reduction in the total dry mass if the mosquitoes were not provided sugar during periods of rehydration (Benoit et al., 2009). Dry mass reduction is likely due to the utilization of nutrient reserves (carbohydrate, glycogen and lipid), and these reductions in nutritional reserves lead to decreased survival after multiple bouts of dehydration/rehydration. Additionally, these reduced nutrient reserves lead to lower egg production. These results imply that each response of *C. pipiens* to dehydration and rehydration requires energy expenditure, and after multiple bouts this can significantly impact the mosquito's nutrient reserves. These results suggest that it may be important to assess the effects of multiple bouts of dehydration on the physiology of blood-feeding arthropods, particularly since this alters egg production.

Differences between populations

Recently, population differences have been implicated as an important variable in the dehydration resistance of blood-feeding arthropods. Most of this work
has focused on Anopheles gambiae (Coluzzi et al., 2002; White et al., 2007; Simard et al., 2009). In A. gambiae, the M and S forms display major differences in dehydration tolerance (Lee et al., 2009). The M form survives significantly longer under dry conditions than the S form (Lee et al., 2009). Possibly, the 2La and/or the 2Rbc chromosomal inversions are responsible for this difference because both inversions have been associated with drought tolerance (Touré et al., 1994; Powell et al., 1999). Gray et al. (2009) demonstrated that the 2La inversion improves dehydration tolerance, particularly during early adulthood. One possibility for the increased dehydration resistance associated with the 2La inversion is that this region is involved in maintenance of cuticular proteins containing the RR-2 consensus (White et al., 2007; Gray et al., 2009). Cuticular proteins containing the RR-2 consenus are extremely important for cuticular hardening and may be upregulated in response to dehydration (Rebers et al., 2001; Zhang and Pelletier, 2008). Additionally, changes in glycogen may be increased to improve dehydration resistance in A. gambiae (Gray et al., 2009), as also demonstrated in D. melanogaster (Graves et al., 1992; Djawdan et al., 1998; Archer et al., 2007). These population differences in A. gambiae may be critical for dry season survival. We still know little about the capability of A. gambiae for aestivation and the dehydration resistance that may be expected to go along with such a potential form of dormancy (Charlwood et al., 2000). Thus, significant differences may exist within species that allow certain populations to colonize more arid habitats.

Classification based on water loss rates

Figure 2 summarizes water loss rates for a number of arthropod vectors during their off-host phase (Fig. 2). The classification system is based on that proposed for terrestrial arthropod by Hadley (1994): hygric = high water loss rates, usually over 2.0%/h, mesic = moderate water loss rates, 0.8-2.0%/h, and xeric = low water loss rate, < 0.8%/h. Certain trends are evident for blood-feeding arthropods: mosquitoes are hygric, with fairly high water loss rates, hemipterans are fairly resistant to dehydration with water loss rates consistently below 0.8%/h, and ticks represent an intermediate having water loss rates that vary from extremely low (0.05%/h) to 1.5%/h. *Rhipicephalus* (= *Boophilus*) annulatus has the highest water loss rate for ticks, and this is likely due to the fact it is a one-host tick species that spends most of its life attached to its host and thus had nearly continual access to blood during most of its life (Needham and Teel, 1991). Fleas likewise have intermediate loss rates. Interestingly, the tsetse fly, unlike other Diptera, falls into the xeric category, thus this higher Diptera clearly differs from lower Diptera such as mosquitoes in relation to its water balance characteristics. It is important to note that water loss rates are not the only factor involved in maintaining water balance: the ability to uptake water from the air or tolerate high levels of dehydration are also influential aspects for establishing habitat preferences in relation to water balance. Additionally, even within groups, such as mosquitoes, significant differences have been noted between even closely related species, and as shown for C. pipiens, there are important differences between diapausing and nondiapausing individuals (Benoit and Denlinger, 2007). Yet, the results presented in Fig. 2 suggest that general categories of water loss rates can be predicted for species that have not yet been evaluated.

Water balance after blood feeding

Diuresis following blood feeding

Blood feeding causes immediate and drastic changes in athropod physiology. Research in this field has been conducted primarily on *Rhodnius prolixus* and mosquitoes. The mass of *R. prolixus* nymphs and adult female mosquitoes increases10-12 and 2-3x, respectively. Although this blood meal represents a excellent source of nutrients, a large portion of this resource contains excess Na⁺ and Cl, along with a surplus of water. Without the ability to reduce this excess liquid, the vectors remain corpulent and, thus, prone to predation. Briefly, this excess water is directly passed through the alimentary canal or is absorbed into the hemolymph, where it is transported into the Malpighian tubules and removed during diuresis as urine. Postprandial diuresis yields a condensed meal leaving mostly blood cells and proteins that are digested quickly, in the case of mosquitoes, or slowly in *Rhodnius* (Lehane, 2005). Diuresis in blood-feeding arthropods is regulated by a suite of antidiuretic and diuretic hormones. A comprehensive review of these control mechanisms, along with general insect diuresis, has been provided by Coast (2001; 2009), Dow and Davis (2003), and Beyenbach (2003). For this review, we present a brief summary of diuresis in blood-feeding arthropods, particularly in *Rhodnius* and mosquitoes.

In renal system of insects, epithelial transport mechanisms in the Malphigian tubules regulate secretion of electrolytes, organic solutes and water, and similar mechanisms control re-absorption occurring in the proximal segments of the Malphigian tubules, hindgut and rectum (Beyenbach, 1995; Spring and Albarwani, 1993; O'Donnell and Maddrell, 1995; Coast, 2001; 2009). The renal activity of insects is impressive, with peak turnover rates processing the volume of all extracellular fluid nearly 200 times per day following blood feeding in *Aedes aegypti* (Beyenbach, 2003). This leads to a urination rate of 60 nl min⁻¹ for *A. aegypti*, allowing the mosquito to quickly void excess fluid volume (Williams et al., 1983; Wheelock et al., 1988).

For many blood-feeding arthropods, including mosquitoes and tsetse flies, condensation of the bloodmeal begins during feeding (pre-diuresis), yielding an increase in the concentration of nutrients in the meal (Clements, 1992). For soft ticks (Argasidae), excess water is secreted from the coxal gland, a specialized structure unique to these ticks. Within minutes of blood feeding, diuretic hormone is released in response to distension of the gut (Maddrell, 1966; Coast et al., 2005). A comprehensive list of diuretic, along with a few identified anti-diuretic, hormones are provided for *Rhodnius* by Coast (2009) and in *Anopheles gambiae* by Riehle et al. (2002). Serotonin and natriuretic hormone act conclusively as diuretic hormones for *Rhodnius* and mosquitoes, respectively (Coast et al., 2005; Orchard, 2006; Te Brugge et al., 2009). One recently identified hormone that seems to be particularly important for mosquitoes is Diuretic hormone 44, which has been documented to have an expression profile paralleling excretion (Jagge and Pietranonio, 2008). Diuretic

hormones stimulate the Malphigian tubules, prompting removal of excess water and Na^+ while preserving K⁺ (Coast, 2009). This likely coincides with the peak phase of diuresis, which yields urine highly concentrated in Na^+ . Eventually, the level of Na^+ begins to decline and the level of K⁺ secretion increases, a phase associated with K⁺ release during blood cell digestion (Williams et al., 1983). After this initial phase of rapid diuresis, mosquitoes enter a stable post-peak phase of diuresis in which secretion levels of K⁺ and Na⁺ become more varied and the urine becomes hypoosmotic, allowing the hemolymph to return to normal, post-feeding osmotic concentrations (Beyenbach, 2003). Diuresis is likely terminated by the combined effect of the end of adominal distension, a signal for secreting diuretic hormone, and the presence of anti-diuretic hormones. At this point, the vector has returned to its nonfeed, host-seeking state.

Cuticle changes during and after blood feeding

Throughout blood feeding and immediately after, changes in the cuticle are fairly common and have been documented in mosquitoes, *Rhodnius* and ticks (Marinotti et al., 2006; Yoder et al., 1997; Andersen and Roepstorff, 2005; Melcón et al., 2005; Togawa et al., 2008). Many of these changes involve preparation for the cuticle to undergo rapid stretching during blood feeding, a process know as plasticization (Bennet-Clark, 1962; Reynolds, 1974). The likely mechanism for plasticization is the breakdown of relatively weak intermolecular bonds between proteins (Hackman, 1975; Hackman and Goldberg, 1987; Reynolds, 1975; Quesada-Allué, 1987). Additionally, the cuticular composition of most blood-feeding arthropods contains a wide variety of resilin proteins that allow stretching of the cuticle. While in this distended state, water loss rates have been documented in the lone star tick, *Amblyomma americanum*, bed bugs, *Cimex lectularius*, and *Aedes aegypti* (Yoder et al., 1997; J. B. Benoit Unpublished observation), and in all three species cuticular water loss rates were nearly 3x higher than in nonfed individuals. Of particular interest is the observation that cuticular lipids increased in abundance after blood feeding, yielding insects that are more resistant to dehydration following a bloodmeal (Yoder et al., 1997; J. B. Benoit Unpublished observation). For *A. aegypti*, only the first bout of blood feeding increases the amount of cuticular hydrocarbons and reduces water loss, with subsequent feeding cycles altering neither the amount of hydrocarbon nor the water loss rate. Thus, cuticular changes immediately after blood feeding usually increase cuticular permeability, but the initial blood meal may provide a source of additional cuticular hydrocarbons that can be used to enhance dehydration resistance during the subsequent off-host phase.

Lessons from transcriptome and proteome studies during blood feeding

Recent transcriptome and proteome studies have examined the responses to blood feeding in mosquitoes (Sanders et al, 2003; Dana et al., 2005), sand flies (Jochim et al., 2008), ticks (Rudenko et al., 2005), tsetse flies (Lehane et al., 2003; Munks et al., 2005), and ceratopogonids (Campbell et al., 2005). Many genes that both increased in expression during blood feeding may be involved in response to water stress caused by the bloodmeal. Table 2 provides a list of genes/proteins that increased during blood feeding and are also known to be important during water stress. Other genes that increased during blood feeding may be involved in the response to excess water, but this list includes only genes previously reported to be involved in arthropod water balance (Benoit, 2009). A majority of genes included in this list are involved in preventing oxidative damage, an injury fairly common during dehydration, rehydration, and overhydration (França et al., 2007). Two other categories of genes that were upregulated during blood feeding include chaperones, particularly heat shock proteins, and those involved in the transport of ions and fluid, particularly aquaporins. The chaperones likely repair proteins damaged during blood feeding, and transport proteins maintain cellular water and ions levels. Lastly, structural changes occur as fluid levels within a arthropod fluctuate, and genes for both cytoskeletal proteins and membrane restructuring are increased during blood feeding. Table 2 thus represents a collection of genes that are likely to be involved in preventing and responding to cellular water stress during blood feeding. Future studies will be needed to directly assess functions for these genes in enabling arthropod vectors to tolerate overhydration and to process water during blood feeding.

Conclusions

Many aspects of water balance, particularly those involved in the transition between blood feeding and off-host physiology, have not been fully elucidated in hematophagous arthropods. With the current wealth of molecular information on blood-feeding arthropods, establishing the underlying molecular mechanism for changes that occur during dehydration and in response to excess water during blood feeding should now be feasible. More studies are needed to address the roles of antidiuretic and diuretic hormones during both the blood-feeding and off-host states, particularly how this suite of hormones coordinates the transition between the two states. How other physiological aspects of the blood-fed state, such as cuticular plasticization and changes in the hydrocarbon composition are initiated and executed remain to be fully explained. The vulnerability of vectors to desiccation suggests the potential to generate new methods to alter the dehydration tolerance of blood-feeding arthropods, thus providing new strategies of control. Additionally, moisture features of the environment offer a key indicator of habitat preference and potential species distribution patterns, thus implying that an understanding of these limitations offers predicative value in defining the potential geographic spread of invasive species, especially in a changing global environment. Thus, determining water balance characteristics for each species, as well as for select populations, is important for establishing survival ability and for predicting potential distribution patterns.

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Class	Order	Family	Common name*
Arachnida	Acarina	Dermanyssidae	Poultry mite
		Halarachnidae	Dog nasal mite
		Macronyssidae	Rat mite
		Rhinonyssidae	Lung mite
		Ixodidae	Hard tick
		Argasidae	Soft tick
Insecta	Diptera	Psychodidae	Sand fly
		Ceratopogonidae	Biting midge
		Culicidae	Mosquito
		Simuliidae	Black fly
		Rhagonidae	Snipe fly
		Tabanidae	Horse fly
		Muscidae	Stable fly
		Glossinidae	Tsetse fly
		Nycteribiidae	Bat fly
		Hippoboscidae	Louse fly
		Streblidae	Bat fly
	Siphonaptera [†]	Ceratophyllidae	Chicken flea
		Ctenophthalmidae	Rodent flea
		Leptopsyllidae	Mouse flea
		Pulicidae	Cat and dog flea
	Lepidoptera	Noctuidae	Vampire moth
	Phthiraptera [†]	Philopteridae	Bird lice
	-	Haematopididae	Ungulate lice
		Hoplopleuridae	Armored lice
		Linognathidae	Pale lice
		Pediculidae	Body lice
		Polyplacidae	Spiny rat lice
		Pthiridae	Pubic lice
	Hemiptera	Cimicidae	Bed bugs
		Polyctenidae	Bat bugs
		Reduviidae	Kissing bugs

Table 1.1. List of representative blood-feeding arthropods. *, denotes the common name for the entire group or the most prevalent groups within the family; †, indicates that there are more families within the order but only the most common families are listed.

Gene	Organism	Citation
Antioxidant	organishi	Citation
Catalase	Aedes aegynti	Sanders et al 2003
	Culicoides sonorensis	Campbell et al 2005
	Glossina morsitans	Lehane et al 2003
	Glossina morsitans	Munks et al 2005
	Lutzomvia longinalnis	Jochim et al. 2008
Cytochrome c oxidase	Anonheles gamhiae	Dana et al 2005
Cytoentonie e oxidase	Culicoidas sonoransis	Campbell et al. 2005
	Lutzomyja longinalnis	Lochim et al. 2008
Cutashrama B450	Aadaa aammti	Sondara et al. 2003
Cytochionie F450	Aeues uegypti	Dame at al. 2005
	Anopheles gambiae	Dana et al., 2005
	Lutzomyta tongipalpis	Jochim et al., 2008
Ferritin	Aedes aegypti	Sanders et al., 2003
	Culicoides sonorensis	Campbell et al., 2006
	Lutzomyia longipalpis	Jochim et al., 2008
Iron-binding proteins	Culicoides sonorensis	Campbell et al., 2005
Glutathione peroxidase	Glossina morsitans	Munks et al., 2005
Glutathione s-transferase	Anopheles gambiae	Dana et al., 2005
	Ixodes ricinus	Rudenko et al., 2005
	Lutzomyia longipalpis	Jochim et al., 2008
Peroxidase	Culicoides sonorensis	Campbell et al., 2006
	Glossina morsitans	Lehane et al., 2003
Peroxiredoxin	Glossina morsitans	Munks et al., 2004
	Lutzomyia longipalpis	Jochim et al., 2008
Superoxide dismutase	Glossina morsitans	Munks et al 2005
	Lutzomvia longinalnis	Jochim et al 2008
	Euromyta tongipulpis	500mm et ul., 2000
Thioredovin reductore	Andre anomiti	Sandars et al. 2003
Thioredoxin reductase	Aronholog gambiag	Dama at al. 2005
	Anopheles gambiae	Campbell at al. 2005
	Culicolaes sonorensis	Campbell et al., 2005
	Glossina morsitans	Munks et al., 2005
	Ixodes ricinus	Rudenko et al., 2005
Chaperone		
Hsc70	Aedes aegypti	Sanders et al., 2003
Hsc70A	Culicoides sonorensis	Campbell et al., 2005
Hsc70B	Culicoides sonorensis	Campbell et al., 2005
Hsp70	Aedes aegypti	Benoit et al., 2009
Hsp82	Anopheles gambiae	Dana et al., 2005
Hsp90	Aedes aegypti	Benoit et al., 2010
smHsp	Anopheles gambiae	Dana et al., 2005
•		
Structural		
Actin	Anonheles gambiae	Dana et al 2005
	Ixodes ricinus	Rudenko et al 2005
	Lutzomvia longinalnis	Jochim et al 2008
	Eucomyna tongipuipis	Joenni et ul., 2000
Transport		
Amino acid transporter	Andas anomiti	Sanders et al. 2002
	Aedes degypti	Sanders et al., 2003
Aquaporin		Jochim et al., 2008
Aquaporin 2	Aeaes aegypti	Sanders et al., 2003
Aquaporin 4	Aedes aegypti	Sanders et al., 2003
Cl ⁻ channel	Aedes aegypti	Sanders et al., 2003
Organic cation porter	Aedes aegypti	Sanders et al., 2003
Na ⁺ /iodine symporter	Anopheles gambiae	Dana et al., 2005
Na ⁺ /multivitamin symporter	Aadas aaminti	Sanders et al. 2002
V ATDaga	Aedes accurti	Sanders et al., 2003
v-n1FdSC	Aeues uegypti	Dana et al., 2005
	Anopheles gamblae	Dana et al., 2005
W/-+	Luizomyia longipaipis	Joenim et al., 2008
water transporter	Anopneles gambiae	Dana et al., 2005
0' I'		
Signaling		0 1
Calponin-like protein	Aedes aegypti	Sanders et al., 2003
Calmodulin-beta	Aedes aegypti	Sanders et al., 2003
GRP94	Aedes aegypti	Sanders et al., 2003
Membrane restructuring		
Enoyl CoA hydratase	Aedes aegypti	Sanders et al., 2003
Fatty acid synthase	Aedes aegypti	Sanders et al., 2003
Fatty acid desaturase	Aedes aegypti	Sanders et al., 2003
Fatty-acid-conenzyme A ligase	Aedes aegypti	Sanders et al., 2003
Short-branch Acyl-CoA debydrogenese	Aedes geownti	Sanders et al. 2003
Short-oranen Acyr-CoA dellydrogendse	neues degypti	Sanders et al., 2005

Table 1.2. Genes and/or proteins that increase during blood feeding and may be involved in responding to excess water from the bloodmeal.



Figure 1.1. Diagrams of the two common types of blood feeding in arthropods. (A) blood feeding common in adult arthropods; (B) blood feeding common for lower developmental stages (nymphs, larvae) of arthropods.



Figure 1.2. Comparison of the water loss rates for off-host adult female hematophagous arthropods. Classification of xeric, mesic, and hygric is based on Hadley (1994). List in order from lowest to highest water loss rates: *Rhodnius* prolixus (Hemiptera; Hadley, 1994), Ornithodoros savigny (Argasidae; Hafez et al., 1970), Amblyomma cajennense (Ixodidae; Needham and Teel, 1991) Hyalomma dromedarii (Ixodidae; Hafez et al., 1970), Cimex lectularius (Hemiptera; Benoit et al., 2007b). Ornithodoros moubata (Argasidae Lees, 1947), Dermacentor andersoni (Ixodidae; Yoder et al., 2007), Amblyomma maculatum (Ixodidae; Yoder et al., 2009), Rhipicephalus sanguineus (Ixodidae; Yoder et al., 2006), Glossina morsitans (Diptera; Bursell, 1960), Dermacentor variabilis (Ixodidae; Yoder et al., 2004), Dermacentor albipictus (Ixodidae: Needham and Teel, 1991), Amblvomma americanum (Ixodidae; Yoder et al., 2006), Ixodes uriae (Ixodidae; Benoit et al., 2007a), Ixodes scapularius (Ixodidae: Yoder and Spielman, 1992), Ixodes ricinus (Ixodidae; Lees, 1946), Xenopsylla confromi (Siphonaptera; Fielden et al., 2002), Rhipicephalus annulatus (Ixodidae; Needham and Teel, 1991), Xenopsylla ramesis (Siphonaptera; Fielden et al., 2002), Ctenocephalides felis (Siphonaptera; Thiemann et al., 2003), Culex pipiens (Diptera; Benoit and Denlinger, 2007), Aedes aegypti (Diptera; Benoit et al., 2009), Aedes albopictus (Diptera; Benoit, J.B. unpublished observation), Anopheles arabiensis (Diptera; Gray and Bradley, 2005), Anopheles gambiae (Gray and Bradley, 2005).

Water management by dormant insects: comparisons between dehydration resistance during summer aestivation and winter diapause.

Abstract

During summer in temperate regions and tropical dry seasons insects are exposed to extended periods with little available water. To counter this dehydration stress, insects have two options. They can either remain active by utilizing mechanisms to function under severe water stress and high temperatures, or they can escape from the stressful environment by exploiting an aestivation mechanism. During aestivation, insects undergo a variety of molecular and biochemical changes to arrest development, reduce metabolism, tolerate high temperatures, and increase their ability to maintain water balance. In this review, I provide a synopsis of known and possible mechanisms utilized by insects to reduce the stress of dehydration during aestivation. Comparative observations of aestivating and diapausing insects are also discussed to assess similarities and differences in the methods used by insects to increase dehydration resistance between these two types of dormancies. Adaptations that alter moisture requirements during diapause (low metabolic rate, increases in osmolytes, shifts in cuticular hydrocarbons, cell membrane restructing) are likely similar to those utilized at the induction and during the maintenance phase of aestivation. Few studies have been conducted on the physiology, particularly the biochemistry and molecular regulation, of aestivating insects, indicating that much

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more research is needed to fully assess water balance characteristics of insects during aestivation. Whether an insect is in diapause or aestivation, behavioral, biochemical, and physiological adaptations are essential for suppressing water loss and enhancing survival in a desiccated state.

Introduction

Most insects are capable of developing and reproducing during short periods each year when conditions are appropriate for their growth (Denlinger, 1986; 2002). When outside of these optimal conditions, insects have an amazing ability to resist and adapt to fluctuations in their environment for short periods, but when these periods of stress are longer, large scale biological changes are needed (Wolda, 1988; Denlinger, 2002; Danks, 2006). The two most stressful times for most insects are winter (freezing temperatures, lack of water due to its presence as ice, and the absence of food resources) and during the peak of summer or during tropical dry seasons (high temperatures, lack of water due to periods of drought, and inadequate quantities of food). A few insects avoid such periods by migrating to favorable locations, but most species are too small, too slow, or too specialized to engage in long distance flights to new habitats. Thus, most species have mechanisms to tolerate extremely long resting periods under stressful conditions, responses characterized as changes in behavior, reductions in metabolism, prevention of temperature stress, and the maintenance of water balance.

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In this section, I provide a comparative synopsis of water balance characteristics that occur when insects undergo summer aestivation to tolerate heat and drought and winter diapause to survive low temperature and lack of water. Particularly, I will address behavioral, biochemical, and molecular responses that are involved in water management. I discuss four categories of response: 1. Suppressing water loss, including cuticle changes, reductions in metabolic rates and loss of water through regulation of spiracular valves, reducing moisture loss in waste products, and behaviorally by aggregating in favorable microhabitats; 2. Increasing the internal water pool by liquid ingestion, water vapor absorption, and/or metabolic water production; 3. Reducing the stress of dehydration by the upregulation of stress-related proteins (e.g. heat shock proteins), increasing protective solutes (e.g. trehalose), and partitioning water unevenly into metabolically active tissues; and 4. Exploiting water loss to become anhydrobiotic. Additionally, I provide a brief discussion for the role of water as a cue to initiate and break dormancy.

Maintenance of water balance

Development, distribution, and survival are dependent on the insect's ability to maintain water balance. Simply stated, water balance can be summarized by Wharton's (1985) general equation:

 $m = m_S - m_T$,

where water mass (m) is controlled by the movement of water into the insect (m_s) and movement of water out of the insect (m_T). Thus, if $m_S > m_T$, the water pool within the insect increases and if $m_T > m_S$, the water pool decreases. The net transpiration rate or water loss rate (m_T) usually relates to where the insect resides, those living in arid environments or environments that lack water for extended periods tend to have low water loss rates (Hadley, 1994; Gibbs, 2002a). Few insects can tolerate either dehydration or overhydration for extended periods. To use chironomids as examples, the Antarctic midge, *Belgica antarctica*, when held at hydrating conditions $(m_S > m_T)$ for extended periods eventually succumbs to severe overhydration (Lopez-Martinez et al., 2009). At the opposite extreme, the sleeping midge, *Polypedilum vanderplanki*, when dehydrated slowly ($m_T > m_S$; Kikawada et al., 2005; Watanabe, 2006) can reduce its water pool to exceptionally low levels, but even in this extreme example a small percent (2-3%) of body water is essential to allow the midge to recover following anhydrobiosis. For an insect to survive and develop within a particular environment, water balance $(m_S = m_T)$ must be achieved.

Mechanisms utilized by insect to suppress dehydration

Cuticular changes

Readers not familiar with the structure of the insect cuticle can refer to comprehensive descriptions by Hepburn (1985) and Hadley (1994). Briefly, the epicuticle, a thin layer at the external surface of the insect, serves as the interface between the insect and the environment, and underneath is the thick procuticle which represents nearly 95% of the cuticle. The procuticle is divided into three sections, the exocuticle, mesocuticle, and endocuticle, all consisting predominantly of a chitinprotein complex. The exocuticle hardens through sclerotization to provide support to the cuticle, the endocuticle remains soft and highly flexible, and the mesocuticle represents a transition between the endocuticle and exocuticle. Though the epicuticle is significantly thinner than the procuticle, it is responsible for regulating most of the water flux through the cuticle. The cuticulin layer (or outer epicuticle) and the inner epicuticle layer (or dense layer) account for most of the epicuticle, and both are impregnated with lipids secreted by dermal glands. These lipids, which are mainly hydrocarbons, serve to waterproof the cuticle. The outer epicuticle is coated with a wax layer that is secreted through channels from cells below the procuticle. Lastly, the wax layer for some insect species is protected from the environment by an external cement layer composed of lipoproteins. Slight variations in the organization, structure, and composition of the cuticle can significantly alter the waterproofing capacity of the insect cuticle.

Water lost through the cuticle accounts for a significant portion of the total water lost by insects (Hadley, 1994; Chown and Nicolson, 2004). Commonly, species that reside in moist habitats have highly permeable cuticles and those that live in more arid areas have cuticles resistant to water flux (Edney, 1977; Hadley, 1994; Gibbs, 1998; Bradley et al., 1999; Gibbs and Matzkin, 2001). Three possible changes in the cuticle occur to alter water loss: 1. procuticle restructuring, 2. reorganization or changes in the epicuticle, particularly the lipids; and/or 3. the presence of a chorion or pupal case.

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Changes in the procuticle have been well documented, but the involvement for this portion of the cuticle in reducing water loss has not been thoroughly studied (Hadley 1994). Some studies have suggested that the structure of the endocuticle may significantly alter the water movement across the cuticle (Machin and Lampert, 1987; Hadley, 1994). Increases in scleritization of the cuticle could increase the density of the procuticle, thus improving water retention (Benoit et al., 2005). There is also recent speculation that cuticular proteins are involved in water regulation, but this has not yet been documented conclusively. Currently, no studies have documented changes in the procuticle during aestivation or diapause.

The best-known suppressor of water loss is cuticular lipids located on the epicuticle, particularly the wax layer (Blomquist et al., 1987; Hadley, 1981; Lockey, 1988; de Renobales et al., 1991; Hadley, 1994; Gibbs, 1998; Gibbs, 2002b). The composition of the epicuticular lipids varies significantly between species, but the dominant constituents are nonpolar, hydrophobic compounds such as hydrocarbons and their derivatives (Blomquist et al., 1987; Hadley, 1994; Gibbs, 1998; Gibbs, 2002b). It has been well-documented that disturbing the epicuticular lipids alters water loss rates of insects and other terrestrial arthropods (Noble-Nesbitt, 1991; Hadley, 1994), thus their importance as a hydro-insulator is well established. Increases in the amount of cuticular hydrocarbons results in lower cuticular water loss rates (Hood and Tschinkel, 1990; Yoder and Denlinger, 1991; Hadley, 1994; Benoit and Denlinger, 2007; Benoit et al., 2008a), and such increases in cuticular lipids have been well documented during diapause for many insects (Yoder and Denlinger, 1991; Benoit and Denlinger, 2007). In addition to changes in the amount of cuticular lipids.

the composition can also change with long-chained, saturated lipids with few methyl side chains acting as a more effective water barrier (Hadley, 1994; Gibbs, 1998; Benoit et al., 2007a). In most cases, the differences in the cuticular hydrocarbons are the consequence of rearing or storing insects under different conditions. For example, summer-acclimated beetles have more long-chain hydrocarbons than winter-acclimated beetles, and cuticular lipids change during thermal acclimation in grasshoppers (Hadley, 1977; 1994; Gibbs and Mousseau, 1994). Thus, it is likely that aestivating insects have modified profiles of epicuticular lipids, but I am not aware of any experiments that have explicitly tested this hypothesis.

Other characteristics that involve the relationship between lipids and water loss are activation energy (E_a) and critical transition temperature (CTT) (Hadley, 1994). These factors define how temperature influences water loss and cuticle changes (Toolson, 1978; Rourke and Gibbs, 1999; Gibbs, 2002b; Yoder et al., 2005a,b). For many years it was assumed that the CTT involved a transition or melting of lipids that increased water loss rates (Wharton, 1985; Gibbs, 1998; Hadley, 1994). Recent observations on CTT indicate that it does not correlate with increased water loss for many insects, is far above the lethal temperature and may be influenced by factors in addition to lipids; in combination these factors indicate that the CTTs may be an artifact that has no ecological relevance (Gibbs, 2002b; Yoder et al. 2005a; 2005b). Thus, the relevance of the CTT and E_a are in limbo, but trends have been observed. Insects with a low E_a are more resistant to water loss with increasing temperatures, and those with a CTT are actively suppressing water loss until a particular temperature is reached (Yoder et al., 2005b; Benoit et al., 2008a). Additional water-proofing barriers can also occur, particularly during diapause and aestivation. The two most common examples are found in insects that are dormant as eggs or pupae. Insects that undergo dormancy as pharate first-instar larvae within the chorion of the egg are extremely resistant to dehydration. This is likely due to the presence of two water-proofing barriers: the egg chorion and the larval integument. This high resistance to water loss has been demonstrated in many insect eggs (Krysan et al., 1977; Yoder et al., 2004; Roberts, 2004; Benoit et al., 2007b) and is particularly striking in mosquito eggs that undergo diapause (Sota and Mogi, 1992). For fly pupae, the puparium serves a similar function as the egg chorion; it provides an additional layer that helps to suppress water loss. Also, it is important to note that the inside of the puparium or chorion may be coated with additional lipids, providing even more resistance to water loss (Yoder et al., 1992).

Respiratory water loss and metabolism reduction

Along with water lost through the cuticle, respiration represents a secondary route accounting for 5-20% of the total water loss for most insect (Hadley, 1994; Chown, 2002). In comparison to hydrophilic species, insects residing in arid regions usually lose a higher proportion of water through respiration, up to 70%, than through their highly water-proofed cuticle (Hadley, 1994; Chown, 2002). During respiration, water is lost rapidly when the spiracles are open due to the humidity gradient within the tracheal system, which is very high, and the external environment, which is very low, thus causing an outward water flux (Hadley, 1994). Water loss through the spiracles can be suppressed by opening within an internal cavity (Ahearn, 1970;

Benoit et al., 2005); such is the case for beetles with fused-elytra or elytra that rarely open, such as in the tenebrionid, *Eleodes armata* (Ahearn, 1970; Cloudsley-Thompson, 2001) and the spider beetle, *Mezium affine* (Benoit et al., 2005). Another mechanism is to locate the spiracles closely together. This method increases the local humidity around the spiracle cluster, which in turn suppresses water loss through interference (Pugh et al., 1988). Both of these structural arrangements retard water flux out of the spiracles.

The most prominent mechanism to reduce respiratory water loss is the most simple, closing the spiracles. By closing or even partially blocking the spiracles, water loss, particularly at low relative humidities, is reduced (Bursell, 1957; Hadley, 1994; Chown, 2002). Discontinuous gas exchange (DSC) can also reduce water loss through the spiracles by limiting gaseous diffusion to short periods when carbon dioxide accumulates to high levels (Lighton et al., 1993; Hadley, 1994). Recent research has questioned the ability of DSC to reduce water loss, indicating that more studies are needed to determine exactly how DSC relates to water loss suppression (Sláma, 1999; Chown, 2002). Some of the arguments against DSC are that it is abandoned during periods of water stress, respiratory water loss only accounts for a small portion of water loss and that ability of DSC to limit water loss is dependent on increasing the $P_{\rm CO_2}$ to the highest levels, which does not occur naturally (Chown, 2002). Arguments for DSC are other factors such as hemolymph pH and oxygen demand may be more critical under dehydrating conditions than water retention due to changes associated with declining hemolymph volume, thus respiration increases during dehydration (Chown, 2002). Even so, without spiracle control water loss rates

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would be higher (Bursell, 1957; Lighton, 1996; Sláma, 1999; Chown, 2002). One of the hallmarks of dormancy is the suppression of metabolic rate, which allows spiracles to remain closed for extended periods (Hadley, 1994; Storey, 2002; Denlinger, 2002). In particular, flight is a period when water is lost rapidly through respiration (Lehmann, 2001; Chown and Nicolson, 2004), and flight is usually reduced during adult diapause (Denlinger, 1986; 2002).

Excretory system

The elimination of waste is another conduit for the loss of water. The alimentary canal is responsible for the internal regulation of salt and water levels. The regions of the alimentary canal that regulate a majority of the fluid levels are the Malphigian tubules and the hindgut, divided into the ileum and rectum. Many studies have focused on the role of the alimentary canal on water and osmotic regulation, as reviewed by Bradley (1985) and Chown and Nicolson (2004). Briefly, water, along with organic molecules, particularly urine and ions, are absorbed from the hemolymph into the upper portion of the Malphigian tubules, and the urine is then actively concentrated in the lower portion of the tubules. The hindgut, particularly the rectum, acts as the primary site for the re-adsorption of water and select solutes. Insects that are dehydrated or reside in dry regions generate extremely hyperosmotic rectal products as a result of efficient water reabsorption, while aquatic species, those living in moist regions, or those feeding on water-diluted fluid have hypoosmotic rectal products. In some larval Coleoptera, Diptera, and Lepidoptera, Malphigian tubules are not free floating, but instead the distal ends of the tubules are physically

attached to the rectum; this arrangement known as the cryptonephridial system allows the feces to be dried extremely efficiently and in some species is associated with rectal water vapor uptake (Hadley, 1994).

Secretion and absorption by the Malphigian tubules and hindgut reabsorption are regulated by neuropeptide hormones: diuretic hormones cause the secretion of water into the alimentary canal, thus increasing the net water loss, while anti-diuretic hormones act in the opposite direction to retain water. Many of the neuropeptides involved in water balance are summarized in papers by Coast et al. (2002), Riehle et al. (2002), Gäde (2004) and Coast (2006). Some key examples are calcitonin-like peptides, corticotropin-releasing factor related peptides (CRF-related), insects kinins, and cardioacceleratroy peptides, which all function as diuretic hormones, and chloride transport-stimulating hormone (CTSH) and an ion-transport process peptide (ITP) that serve as anti-diuretics (Coast, 2006). The presence and absence of these hormones regulate urine production and subsequent re-aborption of the lost water. Studies involving aestivation and diuretic/anti-diuretic hormones are currently lacking, but I suspect such hormones, particularly anti-diuretics, may be involved in regulating water loss during dormancy.

Nitrogenous waste

Uric acid is the dominant nitrogenous waste product produced by terrestrial arthropods, but guanine and other closely-related nitrogenous products are used by ticks and spiders (Hadley, 1994; Benoit et al., 2008b). Why use uric acid rather than urea or ammonia? Ammonia is highly toxic and highly soluble, thus requiring insects
to immediately expel this waste product, a response that would require large quantities of water; this metabolic end product is thus usually restricted to aquatic insects. Although urea is significantly less toxic than ammonia, it still has to be eliminated in solution. Increases in urea have been linked to increased temperature resistance (Storey, 2004), thus accumulation of this molecule may be useful under certain environmental situations. Uric acid is the least toxic of the potential waste products for insects, and due to its low solubility, excretion of a nearly dry waste product is possible. Also, due to its low toxicity, uric acid can be accumulated within the body of the insect (storage excretion), a situation that completely prevents a loss of water by defecation. This is especially important for insects that aestivate as eggs or pupae since defecation does not normally occur during these stages. Uric acid accumulation occurs in adult insects as well, and has been documented in a few types of bugs, including the shield bug, Parastrachia japonensis, during diapause (Kashima et al., 2006). Some insects contain microbes that generate uricase, an enzyme that breaks down uric acid, thus allowing uric acid to be recycled (Sasaki et al., 1996; Kashima et al., 2006). The reduced metabolism associated with dormancy also means that less nitrogenous waste is produced, thus insects in dormancy generally defecate less or not at all and if they do defecate they utilize uric acid, two features that maximize water conservation.

Behavioral changes

Since aestivation is characterized by heat and water deprivation, changes in insect behavior are frequently involved in the response. The simplest response is to

move into a more favorable region or to hide in protective harborages until favorable conditions return (Denlinger, 1986). Long-distance migrations occurs in only a few insect species (e.g. Monarch butterflies), thus retreating to a near-by protected refugia is the most common response. Within these protective sites the relative humidity is usually higher and the temperature lower, both of which reduce water loss. The protective capacity of these sites can be enhanced by construction of a nest or by lining their microhabitat with silk or wax (Roubik and Michener, 1980; Denlinger, 1986; Kikawada et al., 2005). In many cases, plants can be altered by insects (e.g. galls) to generate small protective harborages (Chown and Nicolson, 2004). Aggregation is also a frequent behavior response associated with dormancy. Formation of an aggregation increases the local humidity, suppressing water loss for members of the group (Yoder et al., 1993; Benoit et al. 2005; 2007b; 2007c). For example, adults of the tropical beetle, Stenotarsus rotundus, form aggregations of up to 70,000 individuals at the base of a palm tree in Panama (Wolda and Denlinger, 1984; Tanaka, 2000). As the group size increases, metabolic rate drops and water conservation is enhanced (Yoder et al., 1993). These benefits gained by forming an aggregation are likely adaptive advantages behind the frequent occurrences of aggregations during periods of aestivation.

Increases in the water pool during aestivation and diapause Ingestion of water

Ingestion of water represents the main route used by terrestrial insect to replenish their water content (Hadley, 1994). Many insects simply drink free

standing water to rehydrate, and this is usually regulated by hemolymph volume (Chown and Nicolson, 2004). For fluid-feeding insects, as with those that live in saline environments, excess ions (K^+ in plant phloem and Na^+ in vertebrate blood and salt water) ingested need to be removed (Wharton, 1985; Hadley, 1994). For blood feeders such as the bed bug, *Cimex lectularius*, blood represents the only source of water between long periods of quiescence (Benoit et al., 2007c). Moisture contained within solid food is sufficient to replace water stores for certain insects. For example, food is the only water source for the spider beetle, *Mezium affine*, a species that survives on the water present in dry stored grain throughout its entire development (Benoit et al., 2005). Some insects retain access to free water during diapause if their habitat temperature remains above freezing and their dormant stage is capable of movement; e.g. females of the northern house mosquito, *Culex pipiens* (Benoit and Denlinger, 2007). But, for many other overwintering species or aestivating species, water may be inaccessible and certain stages such as eggs and pupae do not have the capacity to imbibe water. Thus dormancy in such stages eliminates drinking as an option. Other insects, particularly tenebrionid beetles, ingest massive amounts of water that may push their water content over 70% before entering extremely dry periods (Hadley, 1994; Zachariassen and Pederson, 2002).

Metabolic water production

Metabolism of food resources generates water and this water source is immediately transferred to the water pool of the insect (Edney, 1977; Hadley, 1994). The amount of water released in this manner is dependent on the biomolecules

utilized: fat releases the most water (1.07 ml/g), followed by carbohydrates (0.56 ml/g)ml/g) and proteins (0.40 ml/g). Even though fat metabolism releases the most water, water is also utilized in the metabolism of fat, thus metabolism of sugar, particularly when stored as glycogen, is the most efficient form of metabolism for releasing water (Loveridge and Bursell, 1975; Hadley, 1994; Chown and Nicolson, 2004). The contribution of metabolic water is rather small for most insects, but for species with extremely low water loss rates and those in flight the amount is proportionally higher. This proportional increase has been noted in a few species of desert-dwelling and dryadapted beetles. For two tenebrionids (*Eleodes armata* and *Cryptoglossa verrucosa*) and a spider beetle (Mezium affine), water generated by metabolism contributed substantially to their water pool during their prolonged exposure to dehydrating conditions (Cooper, 1985; Benoit et al., 2005). Metabolic water production can be increased only if ATP accumulation can be reduced (Hadley, 1994). This can be achieved by decoupling within the mitochondria to prevent the conversion of ADP to ATP, but this has been demonstrated only in wax moth larvae (Galleria mellonella; Jindra and Sehnal, 1990) and is not considered by Hadley (1994) to be true for most insects. During periods of aestivation, water is unavailable for extended periods, thus I assume utilization of metabolic water is especially important at this time, yet there are actually few examples documenting the use of metabolic water by terrestrial arthropods during dormancy periods (Dautel, 1999; Hadley, 1994; Danks, 2000). Most likely the paucity of examples reflects the fact that this question has not been adequately addressed. Also, since it is more efficient to retain water than generate water metabolically, increasing metabolism solely to generate water would deplete

food reserves that are critically needed to survive extended dormant periods (Danks, 2000). Thus, I suspect that water generated during aestivation is likely a small but vital by-product of metabolism that contributes to the water pool during dry periods.

Water vapor absorption

Quite a few arthropods, including acarines, beetles, fleas, grasshoppers, walking sticks, flies, lice and isopods, are able to absorb water from subsaturated air (< 99% RH, Hadley 1994). A relatively complete list of arthropods capable of absorbing water vapor from the air is presented by Edney (1977), with later examples listed by Hadley (1994). Water vapor absorption is particularly important for dormant stages such as eggs, pupae and other stages that lack the ability to ingest free water (Danks, 2000). Examples include diapausing flesh fly (S. crassipalpis) pupae (Yoder and Denlinger, 1991), walking stick (Extatosoma tiaratum) eggs (Yoder and Denlinger, 1992) and larvae of the ectoparasitoid, Nasonia vitripennis (Yoder et al., 1994). Although S. crassipalpis and N. vitripennis are both examples of species with temperate zone diapause, the example of the walking stick diapause is a subtropical example with an aestivation diapause, and indeed this species is able to absorb water vapor at relative humidities as low as 30% RH, the lowest reported critical equilibrium humidity (CEH, lowest threshold where water movement into and out of the insect is equal). Water vapor absorption is either active (requiring energy input) or passive (Hadley, 1994; Bayley and Holmstrup, 1999; Danks, 2000). It is important to note that passive absorption occurs at all relative humidities and requires no energy, but it cannot counter water loss unless the relative humidity is at saturation (100%)

RH; Hadley 1994; Chown and Nicolson, 2004). Potential sites utilized by insects for active water vapor absorption are the mouth and anus, and rarely water may be absorbed directly through the cuticle (Hadley, 1994; Bayley and Holmstrup, 1999). Oral and anal sites utilize hyperosmotic or hygroscopic secretions to absorb water (Knülle, 1984; Hadley, 1994). In the case of Collembola, absorption through the cuticle involves the accumulation of osmolytes (Bayley and Holmstrup, 1999). Thus far, the mechanisms of water vapor absorption have been most extensively studied for non-dormant insects, but it is likely that similar mechanisms observed will be evident in aestivating or diapausing insects. Larvae, particularly beetle and lepidopteron larvae, utilize rectal uptake mechanisms. (Edney, 1977; Hadley, 1994). Adult insects predominantly use oral mechanisms (Hadley, 1994). Exactly how eggs and pupae absorb water vapor is currently unknown. Possibly pharate larvae and adults ingest water that has condensed on the inside of the chorion or puparium, but this remains speculation. Thus, the only aestivating insect known to absorb water from subsaturated air is the Australian walking stick, E. tiaratum, which aestivates in the egg stage but quite likely many more examples will be found as the study of insect aestivation expands.

Mechanisms to reduce water stress

Protein and Molecular Changes

Protein and molecular responses common during dehydration are likely essential to insects during aestivation, but few insect studies have focused on this topic. In terrestrial snails, several stress-related proteins (Heat shock proteins, etc.) change in abundance as a result of aestivation and appear to prevent dehydrationinduced stress and mortality (Reuner et al., 2008). Insects may have similar responses during aestivation, but little is known. It is likely more efficient to upregulate proteins to prevent damage rather than repair damage after it has already occurred (França et al., 2007; Lopez-Martinez et al., 2008; 2009).

Heat shock proteins (Hsps) are among the most studied proteins in relation to water balance (Tammariello et al., 1999; Bayley et al., 2001; Hayward et al., 2004; Sinclair et al., 2007; Lopez-Martinez et al., 2008; 2009). Three types of Hsps (smHsp, Hsp70, and Hsp90) have been linked to dehydration and diapause in insects, but no studies have been conducted on aestivating insects thus far. Many insects produce Hsps continually during diapause (Rinehart et al., 2007), but whether this is a component of aestivation is unknown. Nondiapausing insects also express one suite of Hsps during dehydration and a different suite during rehydration (Hayward et al., 2004; Lopez-Martinez et al., 2009). The increase of Hsps either prevents stress damage by acting to prevent unwanted biochemical interactions, by repairing protein damage or prompts the disassembly of proteins damaged by dehydration (Parsell and Lindquist, 1993; Feder and Hofmann, 1999; Rinehart et al, 2007). Recently, we have shown that Hsps are essential for insects to reach their normal dehydration tolerance by blocking their expression with RNA interference (Benoit et al., 2009b). Late embryogenesis abundant proteins (LEAs) seem to act by stabilizing the structure of proteins as the water content within the insect declines (Kikiwada et al., 2008). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), are elevated during dehydration, presumably to reduce damage from oxygen radicals formed from

desiccation-induced stress (França et al., 2007; Lopez Martinez et al., 2008; 2009). Changes in proteins involved with the membrane and cytoskeleton may be fairly common in relation to dehydration (Li et al., 2009). One of the main functions of proteins involved with the cell membrane is to restructure the membrane to reduce water movement out of the cells as the hemolymph osmolality changes. Cytoskeletal protein changes serve to stabilize the cells during the pressure and size changes caused by the osmotic stress of dehydration (Li et al., 2009).

Osmolality, solutes and regulation of size and volume

Insect hemolymph osmolality, measured in mOsm kg⁻¹, ranges between 100 to 1400 mOsm kg⁻¹ and for most insects is around 400-500 mOsm kg⁻¹ (Hadley, 1994). It is important to note that increasing osmolality, even 2-3x, reduces water loss only slightly, and the net water flux out of the insect persists unless the individuals are in an environment near saturation or above their CEH (Willmer, 1980; Chown and Nicolson, 2004). The alimentary canal efficiently regulates ion content, and usually maintains osmolality within 200-300 mOsm kg⁻¹ range for most insects that reside in mesic and xeric regions, without causing internal damage. In some cases, insects sequester ions as insoluble forms, usually in the fat body, during dehydration and these ions can be release when the hemolymph volume increases during rehydration, effectively buffering the osmolality changes within the insect (Chown and Nicolson, 2004). Additionally, combining free fatty acids and amino acids into large insoluble molecules can be used to regulate osmolality. Poor osmoregulators usually have osmolalities that vary nearly 1000 mOsm kg⁻¹ (Hadley, 1994; Elnitsky et al., 2008),

and such insects usually reside in moist microhabitats. Osmolality fluctuations are influenced by a variety of molecules, including, but not limited to, salts (NaCl, KCl), polyols (glycerol), sugars (trehalose), free amino acids (proline, etc.), and free fatty acids.

Some of these molecules that increase during dehydration have protective qualities (Goyal et al., 2005; Benoit et al., 2009a). Trehalose and glycerol are two of the most common molecules that can suppress water loss and reduce stress (Yoder et al., 2006; Watanabe, 2006). Trehalose is especially important during severe dehydration by preventing unwanted protein interactions, decreasing metabolism by altering fluid dynamics and protecting proteins and cellular membranes (Crowe et al., 1992; Suemoto et al., 2004; Goyal et al., 2005; Yoder et al., 2006; Benoit et al., 2009a). Additionally, it is important to note that glycerol and trehalose act to prevent heat and cold stress, resulting in cross-tolerance between temperature and dehydration stress (Yoder et al., 2006; Benoit et al., 2009a). Proline, as a free amino acid, may have similar effects (Yancey, 2005; Ignatova and Gierasch, 2006). This amino acid has been documented to increase during stress in a few insects (Michaud and Denlinger, 2007; Michaud et al., 2008), but future studies are needed to determine the exact function of proline within insects during dehydration. Dehydration-induced changes have also been documented for glucose and sorbital (Hadley, 1994; Elnitsky et al., 2008). Currently, only two studies have looked for changes in protective osmolytes during aestivation. In the case of the blossom weevil, Anthonomus pomorum, trehalose was increased (Koštál and Šimek, 1996), but no changes in

osmolytes were noted in Mediterranean tiger moth, *Cymbalophora pudica* (Koštál et al., 1997).

Another factor that contributes to regulation of osmolality and water content is volume regulation and/or compartmentalization (Zachariassen and Einarson 1993, Hadley 1994, Zachariassen and Pedersen 2002). For example, a significant portion of water may be lost from one water pool (i.e. the hemolymph), but water in another region of the body (e.g. salivary glands and midgut) may remain relatively constant. Typically, water in the tissues is conserved at the expense of the hemolymph. This is particularly intriguing since the osmolality of the hemolymph is usually higher than within the cells during dehydration. Even though the exact mechanisms for retaining water in a tissue at the expense of the hemolymph are not known it is extremely important in order to retain the integrity of biologically active tissue in the body. Overall, much of the stress induced by dehydration can be reduced by regulating osmolality and water pools of the insects.

Body size change represents a mechanism that can increase dehydration tolerance of insects. A key example is provided by the experiments of Gibbs and colleagues on adaptations of *Drosophila* for dehydration resistance. In these studies, dehydration was used as a selective mechanism for *D. melanogaster*, and it was determined that increased water content (over 30%), along with respiratory changes, were the factors that increased dehydration resistance (Gibbs et al., 1997; Folk et al., 2001). Of interest, is the fact that size changes are not present in cactophilic, desertadapted *Drosophila* when compared to mesic species, thus raising the question whether size changes are ecologically relevant or can only be used for inter-species

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comparisons (Gibbs and Matzkin, 2001). Size changes have also been noted during dormancy in insects. The size of the northern house mosquito, *C. pipiens*, increases substantially in preparation for the winter (Benoit and Denlinger, 2007). Interestingly, the water pool does not increase; instead this represents an increase in the dry mass, but even so, the decrease in the surface area to volume ratio reduces water loss (Benoit and Denlinger, 2007). Increasing body size would be a fairly simple mechanism for increasing dehydration resistance in aestivating insects.

Membrane restructuring

As mentioned in section 1.5.1, membrane changes can result in response to dehydration or in preparation for dormancy (Holmstrup et al., 2002; Tomčala et al., 2006). Only a few such studies have been conducted in general and even fewer on insects. Of interest, dehydration-induced changes are minor in comparison to membrane restructing that results from the induction of dormancy (Michaud and Denlinger, 2006; Tomčala et al., 2006). Nearly all of these studies have focused on cold tolerance or the diapause syndrome (Koštál et al., 2003; Michaud and Denlinger, 2006; Tomčala et al., 2006), but I expect that such changes also occur during aestivation. Most responses during diapause involved changes in glycerophospholipids that improve membrane fluidity and improve cold tolerance.

Extreme dehydration tolerance (Anhydrobiosis)

Anhydrobiosis as a mechanism to survive dry seasons deserves special attention. A loss 30-40% of body water content causes death in most insects (Hadley 1994). However, a few species can survive even after losing over 70% of their water content, and anhydrobiotic species can lose over 95% of their water content and still survive (Crowe et al., 1992; Danks, 2000; Watanabe, 2006). Most organisms capable of anhydrobiosis are relatively small, such as tardigrades, springtails and chironomid larvae (Watanabe, 2006). Nearly all insects capable of losing over 70% of their water content are larvae within the midge family, Chirnomidae (Suemoto et al., 2004; Watanabe, 2006; Benoit et al., 2007a). Thus, the remaining discussion in this section focuses predominately on the mechanisms used by chironomid larvae to tolerate extreme dehydration.

For midge larvae to tolerate high levels of dehydration, a particular sequence of changes has been noted. First, water loss has to occur at a relatively controlled rate, which is done so by decreasing cuticular permeability, aggregating, or building structures to reduce water loss (Watanabe, 2006). After dehydration reaches a certain point, high concentrations of certain ions trigger the synthesis of trehalose and other protective molecules such as glycerol and free amino acids (Watanabe, 2006). Slow dehydration yields more osmolytes than rapid dehydration, which is the result of more time available to respond to dehydration stress (Kikawada et al., 2005; Benoit et al., 2007a; 2009a). Within the insects, aquaporins regulate water movement between compartments (Kikawada et al., 2008). These channel protein seems to be

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particularly important in tandem with cells accumulating trehalose, ensuring that vitrification and water-replacement occurs properly. Additionally, LEA and Hsp genes are upregulated during this time to avoid protein denaturation and aggregation (Watanabe, 2006; Kikawada et al., 2008). Throughout this entire process, metabolism is suppressed to prevent oxidative stress and respiratory water loss (Benoit et al., 2007a; Lopez-Martinez et al., 2008). Oxidative stress proteins such as catalase and superoxide dismutase may be increased to reduce dehydration-induced oxidative stress. Normal biological activity is resumed once larvae become rehydrated due to the presence of liquid water.

Overall, this ability to tolerate complete dehydration allows larvae to reside in seasonal water pools. When the dry season begins, these habitats quickly desiccate and the larvae respond accordingly. These responses by the larvae to dehydration prevent subsequent damage from heat and other stresses (Watanabe, 2006; Benoit et al., 2009a). For *P. vanderplanki*, this cross tolerance is extremely important with temperatures in the African dry season pools reaching 60-70°C (Watanabe, 2006). Cross tolerance is also evident in *B. antarctica*: dehydrated larvae are more tolerant to high Antarctic temperatures (30°C; Benoit et al., 2009a). Thus, high levels of dehydration allow chironomid larvae to survive a range of environmental stresses.

Water as a developmental cue

Moisture serves as a cue for breaking aestivation in a number of tropical insects after a long period of water scarcity (Masaki, 1980; Denlinger, 1986; Pires,

2000). Although water may be commonly used to break aestivation, it is used less commonly to initiate aestivation. Waiting until the dry season arrives may be too late to program the entrance into dormancy. Thus, other triggers such as temperature or photoperiod serve more commonly as the cues for the onset of aestivation. One key aspect is that a single, short exposure to water or high humidity does not usually cause termination of dormancy; termination more frequently requires a sustained exposure to wet conditions (Denlinger, 1986; Watanabe, 2006). Brief rains, which are not infrequent during the dry season, can replenish water stores, but using a brief rain to terminate aestivation could be disastrous if the favorable conditions prompted by the onset of rainy season have not been initiated. One way to prevent this accidental break is by suppressing moisture-triggered development until a fixed period of latency has elapsed (Denlinger, 1986; Pires et al., 2000).

Conclusions and future directions

Since maintaining water balance is such a high priority for insects, it is not surprising that a variety of methods are used to accomplish this task, as shown in Table 1. As Table 1 indicates many of the same methods are likely used during aestivation and winter diapause, and I suspect that responses during aestivation and winter diapause will be seen as even more similar when more experiments have been completed. Why are the responses of diapausing and aestivating insects so similar? During winter (diapause) and summer (aestivation), the stresses in respect to moisture requirements are similar: a lack of free water and food resources and low ambient relative humidity. The only major difference is temperature. While temperatures are low during the winter resulting in lower water loss rates, aestivating insects are exposed to high temperatures, which further reduce their ability to retain water. Additionally, only certain biological factors can be altered without expending considerable energy, thus, overlap between the responses of aestivation and diapause is even more likely. Due to higher temperatures, aestivation, particularly during the dry seasons in tropical regions, is likely a more challenging period to maintain water balance than winter diapause even though both types of dormancy use similar methods to increase dehydration resistance.

The paucity of research on insect aestivation indicates a clear need for more focus on this topic if we are to understand the mechanisms involved in water balance of dormant insects. Particularly, studies that focus on environmental cues that trigger aestivation-based changes in behavior and physiology are required. This is not an easy task. To use insect diapause as an example, many groups have focused on triggers, changes and termination of diapause, but considerably more research is still needed to elucidate the fine points of these mechanisms (Denlinger, 2002). With the considerable overlap noted in the water requirements of winter diapausing and aestivating insects, it is likely that other physiological similarities exist. Based on this observation, it is reasonable to use research on winter diapause as a baseline for expanding research on aestivation. Previous papers by Denlinger (2002) and Koštál (2006) discuss changes in diapause that could be utilized for comparative work on aestivation.

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Responses of other aestivating organisms, such as snails, anurans, and fish (Storey, 2002; 2004) have been studied more thoroughly, and offer potential insights that may also be applicable to insect aestivation. To reduce water stress, mechanisms such as reduced respiration and metabolism, increased cuticle barriers, changes in osmolality, and the ability to tolerate losing a high proportion of water content are used by organisms such as snails and anurans (Storey, 2002). Many proteins have been studied for non-insect aestivating organisms (Storey, 2002), and comparative studies could be beneficial for elucidating protein expression themes for insects.

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	Aestivation	Diapause
Supression of water loss		
Reduced respiration	+	+
Spiracle control	+	+
Increased cuticular lipids	(+)	+
Cuticle modifications	(+)	+
Presence of 2nd barrier	+	+
Size increases	(+)	+
Dry waste	+	+
Water intake		
Water vapor absorption	(+)	+
Metabolic water production	+	+
Ingestion of free water	+	+
Stress-reduction		
Proteins		
Heat shock proteins	(+)	+
Late embryogenesis abundant	(+)	(+)
Antioxidant	(+)	+
Cytoskeletal	(+)	+
Protective or colligative solutes		
Glucose	+	+
Glycerol	+	+
Proline	(+)	+
Sorbital	(+)	+
Trehalose	+	+
Membrane alterations	(+)	+
Osmolality regulation	+	+

Table 1.3. Factors that influence water balance characteristics during dormant periods. + indicates it has been documented, (+) indicates it has not been demonstrated, but it will likely be established based on related insect physiology studies.

Chapter 2: Water balance of mosquitoes is affected by two heat shock proteins

Abstract

This study examines the responses of heat shock proteins (Hsp70 and Hsp90) to dehydration stress in three mosquito species, Aedes aegypti, Anopheles gambiae and *Culex pipiens*. We first defined the water balance attributes of adult females of each species, monitored expression of the *hsp* transcripts in response to dehydration, and then knocked down expression of the transcripts using RNA interference (RNAi) to evaluate the potential functions for the Hsps in the maintenance of water balance. Fully hydrated females of all three species contained nearly the same amount of water (66-68%), but water loss rates differed among the species, with A. aegypti having the lowest water loss rate (2.6%/h), followed by C. pipiens (3.3%/h), and A. gambiae (5.1%/h). In all three species water could be replaced only by drinking water (or blood). Both A. aegypti and C. pipiens tolerated a loss of 36% of their body water, but A. gambiae was more vulnerable to water loss, tolerating a loss of only 29% of its body water. Dehydration elicited expression of *hsp70* in all three species, but only *C*. *pipiens* continued to express this transcript during rehydration. *Hsp90* was constitutively expressed and expression levels remained fairly constant during dehydration and rehydration, except expression was not noted during rehydration of C. pipiens. Injection of dsRNA to knock down expression of hsp70 (83% reduction) and hsp90 (46% reduction) in A. aegypti did not alter water content or water loss rates,

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but the dehydration tolerance was lower. Instead of surviving a 36% water loss, females were able to survive only a 28% water loss in response to RNAi directed against *hsp70* and a 26% water loss when RNAi was directed against *hsp90*. These results indicate a critical function for these Hsps in mosquito dehydration tolerance.

Introduction

Insect responses to dehydration are defined by two factors: maintaining the water balance necessary for activity and reducing stress from fluctuations of the internal water pool (Hadley, 1994). To maintain water balance, insects must both replenish water and prevent excessive water loss through retention. Water uptake is accomplished by ingesting water, absorbing water vapor, or the production of metabolic water (Hadley, 1994). Water loss is retarded by improving the water-proofing capacity of the cuticle, increasing water re-absorption in the alimentary canal, reducing respiratory water loss, and/or altering behavior (Gibbs, 1998; Gibbs et al., 2002; Chown and Pederson, 2004). Stress reduction is accomplished by increasing protective osmolytes, such as trehalose, glycerol and proline, and the up regulation of stress proteins, particularly heat shock proteins (Benoit, 2009). Several studies have focused on the dehydration-resistance of adult mosquitoes (Gray and Bradley, 2005; Benoit and Denlinger, 2007), but little is known about the relationship between heat shock proteins and water stress.

In this study, we examined the water balance characteristics of three mosquito vectors of disease: *Aedes aegypti* (dengue, Chikungunya, and yellow fever),

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Anopheles gambiae (malaria), and *Culex pipiens* (West Nile virus). How these mosquitoes tolerate dehydration is important to public health throughout the world since this physiological characteristic is linked to habitat preferences and activity (Hadley, 1994; Benoit, 2009). To examine water balance requirements we determined water content, water loss rates, dehydration, and we also monitored expression levels of transcripts encoding Heat shock protein 70 (Hsp70) and Hsp90 during dehydration. These two Hsps are upregulated in response to a wide range of environmental stresses, (Parsell and Lindquist, 1993; Feder and Hofmann, 1999; Rinehart et al., 2007), including dehydration (Tammariello *et al.*, 1999; Hayward *et al.*, 2004). Finally, we knocked down expression of *hsp70* and *hsp90* in *A. aegypti* to evaluate the functional role for these genes in the maintenance of water balance.

Materials and Methods

Mosquitoes

A. gambiae (Suakoko strain), *A. aegypti* (Rockefeller Strain), and *C. pipiens* (Buckeye strain) were reared according to standard protocol previously described (Gary and Foster, 2004; Mostowy and Foster, 2004; Robich and Denlinger, 2005). Briefly, all three species were held at 27°C under long daylength (16:9 L:D) to prevent diapause and to standardize rearing conditions (Robich and Denlinger, 2005). Larvae were feed a diet of ground fish food (Tetramin). Adults were provided access to 10% sucrose and water ad libitum. Adult females were used 8-10d post-ecdysis unless otherwise noted. Adults utilized for RNAi were injected 5-7d post-ecdysis to begin water balance experiments 8-10d post-ecdysis, i.e. 3 days post-injection.

Water balance characteristics

Mass changes in the mosquitoes were monitored gravimetrically using an electrobalance (CAHN 35, Ventron Co., Cerritos, CA). Each mosquito was weighed singly without enclosure after a brief CO₂ knockdown according to Benoit and Denlinger (2007). Mosquitoes were transferred to the weighing pan and returned to the experimental conditions within 1 min. Relative humidities were generated using saturated salt solutions (Winston and Bates, 1960), 0% RH was established with calcium sulfate, and double-distilled water was used to create 100% RH. All test relative humidities were validated with a hygrometer (Taylor Scientific, St. Louis, MO). All observations were conducted at 22-24°C in an environmental room to allow comparison to previous water balance literature (Hadley, 1994; Benoit and Denlinger, 2007).

The amount of water available for exchange (m, water mass) was determined according to standard methods for mosquitoes (Wharton, 1985; Hadley, 1994; Benoit and Denlinger, 2007). Mosquitoes were placed at 0%, 22-24°C until a loss of 4-6% of their mass to ensure that mass changes reflected a shift in the water pool (Wharton, 1985). Subsequently, consecutive mass determinations (0% RH, 22-24°C) were made at hourly intervals for a total of six mass readings, and then individuals were transferred to 80°C, 0% RH to determined dry mass (d, denoted by five consecutive daily mass measurements with no changes). Water mass was determined by subtracting dry mass from the initial mass. Initial, immediate and final water mass values were analyzed according by Wharton (1985) for determining water loss rates:

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where m_t is the water mass at any time t, m_0 is the initial water mass, and k is the rate of water loss expressed as %/h. The dehydration level at which the mosquitoes can no longer right themselves and fly when prodded was defined as the critical activity point. This denotes the dehydration tolerance limit, an irreversible lethal amount of water loss.

Drinking of liquid water was tested by allowing mosquitoes access to Evans blue (0.1%) dyed water for 24h. The mosquitoes were dissected in 10% NaCl using a light microscope and examined for the presence of blue coloration (Benoit and Denlinger, 2007). Water vapor was tested as an additional resource by measuring mass changes of mosquitoes held at multiple relative humidities (33, 75, 85, 93, 98 and 100% RH) every 12h until death. Maintenance of a stable water pool at any relative humidity, particularly below saturation, is evidence water loss is countered by water gain, and the lowest relative humidity where a stable water pool occurs is denoted as the critical equilibrium humidity (CEH). A lack of water uptake at relative humidities below saturation indicates the CEH is above the water activity within the insect (0.99a_w), thus the CEH = 100% RH (Wharton, 1985).

Cloning and northern blots

Hsp70 was cloned from all three mosquitoes using the TOPO TA cloning kit (Invitrogen, Carsbad, CA). Species-specific primers were designed from sequences found in online databases (GenBank, Vector Base). Hsp90 was also cloned from all three species in the same manner. The control gene, 28S, was cloned using homology primers designed for dipterans (forward primer 5'-CTGTGGATGAACCAAACGTG-3'; reverse primer 5'-TGTACGCCAGCGGTAATGTA-3'). All three genes for the three species were matched (100%) to original sequences in the online databases.

Total RNA was extracted from groups of twenty adults using Trizol reagent (Invitrogen, Carlsbad, California). RNA (eight micrograms per treatment) samples were run on a 1.4% agarose, 0.41 M formaldehyde gel. The RNA was then transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ) with the Schleicher & Schuell's Turboblotter transfer system. Labeled DNA clones for hybridization were generated overnight at 38°C with a Roche Diagnostic's DIG-High Prime labeling kit. Northern blots were prepared according to the DNA Labeling and Detection Starter Kit II (Roche) and exposed to Blue Lite Film (ISC BioExpress, Kaysville, Utah) for 30 min. Membranes were stripped and rehybridized with 28S (control), and all northern blots were performed as technical triplicates.

dsRNA preparation and injection into mosquitoes

Hsp70 and Hsp90 dsRNA was prepared using a MEGAscript T7 transcription kit (Ambion, Austin, TX) according to Sim and Denlinger (2008). PCR-derived fragments were generated from primers (5'-

TAATACGACTCACTATAGGGCAGGCCGATATCAAACACTGG-3' and 3'-TAATACGACTCACTATAGGGCCCAGCTGTGGCTCTTACCTC-5' for Hsp70

and 5'-TAATACGACTCACTATAGGGCAAGCTCCTCGTTGAAAAGG-3' and 3'-TAATACGACTCACTATAGGGCTTCAGGTAGTCGGGGGATCAG-5' for

Hsp90). Sequences obtained were blasted to those from *A. aegypti* for validation of Hsp70 and Hsp90. For the knockdown experiments approximately 0.8-1.0 µl dsRNA for Hsp70 ($1.1\mu g/\mu l$) or Hsp90 ($1.2\mu g/\mu l$) was injected into the thorax of coldanesthetized female mosquitoes using a microinjector. Controls consisted of 0.9-1.0 µl injections of dsRNA of β-galactosidase (β-gal; $1.1\mu g/\mu l$). Thus, for all treatments mosquitoes were injected with approximately $1\mu g$ dsRNA. The selected concentrations and injection volumes were based on previous studies using mosquitoes of similar size (Sim and Denlinger, 2008) and optimization experiments. Three days after injection, expression levels of *hsp70* and *hsp90* were evaluated using northern blots after exposing injected and control mosquitoes to dehydration at 0% RH for 5h. Females that lost 25% of their water content were tested for expression, and this was replicated three times. After verification of *hsp70* and *hsp90* knockdown, water content, survival during exposure to 0% RH, water loss rates, and dehydration tolerance were measured for RNAi-injected females.

Statistics

Each water balance experiment was replicated three times with 20 mosquitoes per replicate. Calculated means \pm S. E. were compared using one- and two-away analysis of variance (ANOVA) followed by the Bonferroni-Dunn test. Percentages were arcsin transformed before analysis. Data from regression lines were assessed by testing for equality of slopes (Sokal and Rohlf, 1995).

Results

Water content

Water balance characteristics for adult females of *A. aegypti, A. gambiae*, and *C. pipiens* are presented in Table 2.1. Water mass correlated with dry mass in all cases ($r^2 > 0.84$), indicating water flux is standardized according to size (Wharton, 1985). In relation to initial mass, *C. pipiens* was the largest of the three mosquitoes, followed by *A. aegypti*, with *A. gambiae* being the smallest (Table 2.1; ANOVA, P < 0.05). As with size, *C. pipiens* contained more water than *A. aegypti* or *A. gambiae*. Percent water content for the three mosquitoes was not significantly different; water content was 66-68% for each species (ANOVA; P > 0.05). Though the mosquitoes are of different sizes, all three species have a similar proportion of body water.

Water loss

Significant differences in water loss rates were noted in the three species (Fig. 2.1; Table 2.1). *Anopheles gambiae* was the most porous, with a water loss rate of 5.1%/h, followed by *C. pipiens* (3.3%/h) and *A. aegypti* (2.6%/h). Rates were significantly different for each species (ANOVA; P < 0.05). *A. aegypti* and *C. pipiens* could tolerate a 34-36% loss of body water (ANOVA, P > 0.05, Table 2.1), but tolerance of *A. gambiae* was significantly lower, 29% (ANOVA; P < 0.05). Based on water loss rates and dehydration tolerances, *A. aegypti* is the most resistance to water loss, and this is evident by its higher survival at 0% RH (Fig. 2.2).

Water gain

Water content declined at all relative humidities below saturation for all three species (Fig. 2.3), indicating that the CEH is at water vapor saturation (100% RH) for these mosquitoes. Water loss declined with an increase in RH, indicating an increase in passive gains, but these gains could not counter water loss at any RH but saturation. As seen previously (Benoit and Denlinger, 2007), water loss increased linearly below 98% RH, but a rapid increase was noted between 100% and 98% RH (Fig. 2.3). To determine if dehydration could prompt water vapor uptake in A. aegypti and A. gambiae (failure to do so was noted in C. pipiens previously; Benoit and Denlinger, 2007), mosquitoes were held at 75% RH until a loss of 10-15% of their water mass was noted; mosquitoes were then transferred to 98% RH. Water masses continued to decline for both mosquito species under these conditions, indicating that dehydration does not trigger an uptake mechanism. Thus, water vapor is not the primary source for replenishing moisture levels. When offered water dyed with Evans blue, dehydrated mosquitoes (10-15% water loss) moved to the periphery of water droplets and inserted their proboscis. After a 24h exposure to water droplets, mosquitoes were removed, killed by exposure to -20°C, and examined for the presence of blue coloration in their crop and midgut. Blue coloration was noted in all cases, indicating that liquid ingestion serves as the primary source used by these mosquitoes to increase their water pool.
Heat shock proteins

Expression patterns for *hsp70* and *hsp90* are presented in Fig 2.4. *Hsp* 70 was expressed during dehydration in *A. aegypti* but not during rehydration. *Hsp90* expression in *A. aegypti* was high in the control indicating constitutive expression and remained high in dehydrated and rehydrated mosquitoes (Fig. 2.4a). Expression of *hsp70* in *A. gambiae* was similar to that of *A. aegypti* (Fig. 2.4b). The pattern was a bit different for *C. pipiens*: both *hsp70* and *hsp90* were expressed during dehydration, but only *hsp70* was expressed during rehydration (Fig. 2.4c).

Hsp70 and Hsp 90 RNAi in A. aegypti

Injection of dsHsp70 and dsHsp90 into A. aegypti reduced the levels of hsp70and hsp90 expression during dehydration > 50% (Fig. 2.5). Sham and $ds\beta$ -gal injections served as controls and did not alter the dehydration-induced expression of hsp70 or hsp90. Thus, expression of hsp70 and hsp90 was substantially reduced in A. aegypti by RNAi. Survival following dehydration at 0% RH was reduced by 2-3 days after injection of dsHsp70 and dsHsp90 compared to those injected with $ds\beta$ -gal (Fig. 2.6a, ANOVA, P < 0.05). dsHsp70, dsHsp90, $ds\beta$ -galactosidase and sham injection had no effect on water loss rates of adult females (Fig 2.6b, ANOVA, P > 0.05). But, dehydration tolerance following injection of dshsp70 and dshsp90 was reduced 6-9% (Fig. 2.6c). Thus, Hsp70 and Hsp90 both appear to be essential for A. aegypti to maintain its maximum dehydration tolerance.

Discussion

Basic water balance characteristics of these three mosquito species are similar to those found in most insect species (Hadley, 1994). The percent water content of the mosquitoes examined in our experiment was 66-68%, a value similar to that of most insects (Hadley, 1994) and similar to values obtained previously in *A. gambiae* (66%; Gray and Bradley, 2005) and *C. pipiens* (66.7%; Benoit and Denlinger, 2007). Dehydration tolerance of these three mosquito species also fell within the range typical of most insects, 25-40% (Hadley, 1994). However, the significantly smaller size of *A. gambiae*, compared to *A. aegypti* and C. *pipiens*, likely makes this mosquito more prone to dehydration as a result of its higher surface area to volume ratio.

Water loss rates of the mosquitoes were relatively high, indicating that these species are preferentially adapted for survival in moist environments (Hadley, 1994; Benoit and Denlinger, 2007). This study, along with the previous study by Gray and Bradley (2005) on *A. gambiae* and *A. arabiensis* and our study on *C. pipiens* (Benoit and Denlinger, 2007), suggests that most mosquitoes will likely be classified as hydrophilic or water sorption dependent in relation to their water loss rates (Hadley, 1994), but more species will need to be examined to establish a solid relationship between mosquito habitat preferences and water loss rates. Survival times of mosquitoes held at 0% RH correlated with water loss rates, and these were similar to those reported by Gray and Bradley (2005) for *A. gambiae* and Benoit and Denlinger (2007) for *C. pipiens*. Thus, based on water loss rates, *A. aegypti* is the most resistant to dehydration, followed by *C. pipiens*, then *A. gambiae*. To counter

their high moisture requirements, these mosquito species likely prefer moist, protective microhabitats and host search only when atmospheric water levels are high or at night when temperatures are lower, features that reduce water loss, a prediction that is consistent with the recorded activity patterns for these species (Kessler and Guerin, 2008). Similar behavior has been demonstrated in other hematophagus arthropods including ticks (Crooks and Randolph, 2006) and *Rhodnius prolixus* (Heger et al., 2006), both of which vigorously search for hosts when their saturation deficits are low.

None of the mosquitoes examined were able to counter water loss at relative humidities below saturation, indicating that they are unable to uptake water vapor from the air below saturation (this study, Benoit and Denlinger, 2007). Additionally, it is important to note that mosquitoes dehydrated at lower relative humidities (*i.e.* 75% RH) and subsequently transferred to higher relative humidities (98% RH) below saturation failed to take up water vapor, thus we have no evidence to suggest that a water vapor uptake mechanism operates in these mosquitoes. As reported by Benoit and Denlinger (2007), water loss only occurred linearly from 0-100% RH, suggesting that mosquito activity may be higher at relative humidities approaching saturation, resulting in increased respiratory water loss at high RHs. To replenish their water stores, all three mosquitoes rely on ingested fluids (free water or blood), indicating that most mosquitoes will need to replenish water orally as adults. Thus, adult mosquito survival is dependent on access to liquid water, either in a bloodmeal or as free water.

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Regulation of Hsps during dehydration was reported in one previous mosquito study that investigated expression of hsp70 in C. pipiens (Rinehart et al., 2006). In our present study, we show that the responses of hsp70 and hsp90 can differ, a difference also noted in the Antarctic midge, *Belgica antarctica* (Lopez-Martinez et al., 2009). Increased expression of hsp70 in response to dehydration was reported in the flesh fly, S. crassipalpis (Tammariello et al., 1999; Hayward et al., 2004), and more recently in the bed bug, Cimex lectularius (Benoit et al., 2009), but expression was not influenced by dehydration in *D. melanogaster* (Sinclair *et al.*, 2007). *hsp90* is constitutively expressed at low levels throughout development in most insects and is elevated in some species by dehydration (Benoit et al., 2009; Lopez-Martinez et al., 2009), but not in others (Hayward et al., 2004). Few studies have been conducted on rehydration in insects, but from the expression patterns that have been observed, species variation is evident. In some cases the same expression pattern observed during dehydration persists during rehydration (Lopez-Martinez et al., 2008; Benoit et al., 2009), but in others different *hsps* respond to rehydration (Hayward et al., 2004). In this study, we show that *hsp70* and *hsp90* are both responsive to the hydration state, albeit with slight differences to dehydration and rehydration in the three mosquitoes we examined.

When RNAi was used to knockdown *hsp70* and *hsp90* expression in *A*. *aegypti*, no differences were noted in the basic water balance characteristics of dry mass, water mass, percent water content and water loss rate. Reduced survival time was noted after injection of *dshsp70* and *dshsp90*, and this was most likely a direct result of a reduction in dehydration tolerance. While controls could tolerate a loss of

36% of their body water, the dsHs70 and dsHsp90 mosquitoes were less tolerant and survived a loss of only 26-28% of their body water. The cellular stress of dehydration intensifies as the amount of water lost approaches the dehydration tolerance limit of 36%. While in this low hydration state, increased protein aggregation and denaturation are likely to occur (Pestrelaski et al., 1993; Goyal et al., 2005), reactive oxygen species may be generated (França et al., 2007; Lopez-Martinez et al., 2009), and phase transitions in membranes may occur, influencing cellular ion homeostasis and altering transmembrane proteins (Crowe et al., 1992; Hayward et al., 2004). We hypothesize that Hsp70 and Hsp90 help ameliorate the stress incurred by cellular desiccation that occurs near the limit of dehydration tolerance, thus resulting in the decreased dehydration tolerance we noted in A. aegypti when the Hsps were knocked down. RNAi directed against Hsps in S. crassipalpis (Rinehart et al., 2007) and the linden bug, Pyrrhocoris apterus (Koštál and Tollarová-Borovanská, 2009) resulted in reduced heat and cold tolerance, but this is the first report demonstrating a functional role for Hsps during dehydration

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	A. aegypti	A. gambiae ¹	C. pipiens ²
Water content			
Initial mass (mg)	2.48 ± 0.11	1.82 ± 0.14	3.61 ± 0.19
Dry mass (mg)	0.83 ± 0.09	0.60 ± 0.12	1.21 ± 0.13
Water mass (mg)	1.65 ± 0.08	1.22 ± 0.11	2.40 ± 0.16
Percent water (%)	66.5 ± 3.2	67.0 ± 2.5	66.5 ± 2.6
Water loss			
Rate (%/h)	2.56 ± 0.19	5.12 ± 0.34	3.26 ± 0.25
Loss tolerance (%)	36.4 ± 2.2	29.0 ± 2.1	34.4 ± 1.8
Survival at 0% RH (h)*	15.3 ± 0.8	5.4 ± 1.1	10.2 ± 0.8
Water gain			
Free water uptake	+	+	+
$CEA(a_v)$	1.00	1.00	1.00

Table 2.1. Water balance characteristics of three female mosquitoes (*Aedes aegypti*, *Anopheles gambiae*, and *Culex pipiens*). CEA, critical equilibrium activity; +, positive occurrence. Superscripts indicate results are similar to those reported by (1) Gray and Bradley (2005) and (2) Benoit and Denlinger (2007). All values are expressed as the mean \pm SE of 45 individuals.



Figure 2.1. Net water loss rates for females of *Aedes aegypti*, *Anopheles gambiae*, and *Culex pipiens*. The slope represents the water loss rate in % h^{-1} . m_t represents the water mass at anytime t and m_0 is the initial water mass. Values represent the mean \pm SE of 45 mosquitoes.



Figure 2.2. Percent survival of adult females of *Aedes aegypti, Anopheles gambiae*, and *Culex pipiens* when held at 0% RH. Each point represents the mean \pm SE of 6 groups of 10 mosquitoes.



Figure 2.3. Water loss rates at relative humidities ranging from complete dryness (0% RH) to water saturation (100% RH) for adult females of *Aedes aegypti, Anopheles gambiae*, and *Culex pipiens*. Rates were determined in the same manner as Fig. 2.1



Figure 2.4. Northern blot hybridizations displaying mRNA expression of *hsp70* and *hsp90* during dehydration in three mosquitoes (*Aedes aegypti, Anopheles gambiae*, and *Culex pipiens*). 28s was used as a control gene. Control, fully-hydrated mosquitoes; 5, 15, and 25% represent percent reduction in water content; rehy, mosquitoes that lost 15% of their water content and were then rehydration at 100% RH in the presence of liquid water. Each blot was replicated three times.



Figure 2.5. Northern blot hybridizations displaying knockdown of *hsp70* and *hsp90* transcripts after injection of *dsHsp70*, *dsHsp90*, and ds β -galactosidase. A sham injection was conducted as an additional control. Each knockdown experiment was conducted three times.



Figure 2.6. Physiological changes in water balance features in adult females of *Aedes aegypti* following injection of *dsHsp70*, *dsHsp90*, and dsβ-galactosidase. A sham injection was also performed to control for possible cuticle damage that may have altered water vapor exchange. (A) Time to 50% mortality for mosquitoes held at 0% RH (B) water loss rates (% h⁻¹) determined according to Fig. 2.1, and (C) dehydration tolerance displayed as percentage of water loss tolerated (mean of three groups of 20 mosquitoes each). *, denotes significance difference from sham control.

Chapter 3: Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*

Abstract

One of the major challenges of overwintering in the mosquito, *Culex pipiens*, is prevention of dehydration. In this study, we compare the water balance requirements of nondiapausing and diapausing adult females of *C. pipiens*. Although their percentage water content is lower, diapausing females contain both higher initial and dry masses than nondiapausing individuals. Both nondiapausing and diapausing females tolerate a loss of up to 40% of their water mass before dying, but diapausing female C. pipiens reach this point after a longer period due to their lower rate of water loss. Males, which do not overwinter in diapause, showed no differences in their water balance characteristics when reared under diapausing or nondiapausing conditions. Likewise, no changes were noted in the water balance of pupae, indicating that diapause-related changes do not occur prior to adult eclosion. This mosquito does not replenish internal water stores by generating metabolic water or by absorbing vapor from the atmosphere, but instead relies on drinking liquid water (or blood feeding in the case of nondiapausing females). The critical transition temperature, a point where water loss increases rapidly with temperature, was the highest for females, then males, then pupae, but was not influenced by the diapause program. Females in diapause did not utilize common polyols (glycerol, trehalose and sorbitol) to retain water, but instead the presence of twice the amount of cuticule hydrocarbons 105

in diapausing compared with nondiapausing females suggests that the deposition of hydrocarbons contributes to the reduced rates of water loss. The laboratory results were also verified in field-collected specimens: mosquitoes in the late fall and winter had a lower percentage water content and water loss rate, higher initial mass, dry mass and more cuticular hydrocarbons than individuals collected during the summer. Thus, the major features of diapause that contribute to the suppression of water loss are the large size of diapausing females (reduction of surface area to volume ratio lowers cuticular water loss), their low metabolic rate and the deposition of extra cuticular hydrocarbons.

Introduction

The northern house mosquito, *Culex pipiens* (L.), a vector of West Nile virus, is established across much of the temperate zones in Asia, Europe and North America (Vinogradova, 2000). To survive winter, newly eclosed females, cued by short daylength and low temperature during the fourth larval instar and as pupae, enter diapause, a stage characterized by hypertrophy of fat reserves from sugar feeding (Mitchell and Briegel, 1989; Bowen, 1992), a halt in blood-seeking behavior (Bowen et al., 1988), an arrest of ovarian development (Sanburg and Larsen, 1972; Spielman and Wong, 1973) and enhanced stress tolerance (Rinehart et al., 2006). Well-protected caves and culverts are used for overwintering. Low temperature and dehydration are among the most significant environmental challenges that diapausing mosquitoes confront during the winter, and it is clear that diapausing females of *C*.

pipiens are better able to withstand these challenges than their nondiapausing counterparts (Rinehart et al., 2006).

In this study, we further explored the enhanced tolerance of diapausing females of C. pipiens to desiccation by comparing the water balance profiles of diapausing and nondiapausing individuals. Water balance occurs when the internal water pools are held at a steady state (water loss = water gain). Mechanisms to prevent dehydration by reducing water loss, such as the accumulation of polyols and the deposition of excess cuticular lipids, were also investigated. Achieving water balance is especially challenging for mosquitoes with their large surface to volume ratio and high respiration rates during flight that yield excessive water loss (Arlian and Ekstrand, 1975; Wharton, 1985). To counter water loss, uptake must occur by either imbibing liquid water, absorbing water vapor from the air or from metabolism. Blood feeding is not an option for diapausing females because they do not take a blood meal until diapause has been terminated (Robich and Denlinger, 2005). How mosquitoes manage their water levels as adults has not been extensively studied and has primarily focused on basic survival studies of adults at various relative humidities (Gray and Bradley, 2005; Rinehart et al., 2006). We conclude from this study that the diapausing female's ability to suppress water loss is the predominant mechanism used by C. pipiens to prevent overwinter dehydration; suppression is achieved by a larger body size of diapausing females, their decrease in metabolism and higher accumulation of cuticular hydrocarbons.

Materials and Methods

Mosquitoes and experimental conditions

The strain of *Culex pipiens* (L.) used in this study was collected from Columbus, Ohio, USA (September 2000, Buckeye strain) and enters diapause when exposed to low temperature (18°C) and short day length (9 h:15 h, L:D) (Rinehart et al., 2006; Robich and Denlinger, 2005). The colony was maintained at 25°C and 70– 75% relative humidity (RH) with a 15 h:9 h, L:D cycle. Adults were provided with honey-soaked sponges and standing water *ad libitum*. Eggs were obtained from 4–5week-old adult females that fed on a chicken and were allowed to oviposit in dechlorinated tapwater. Larvae were held at a density of approximately 250 individuals in a 18 cm x 25 cm x 5 cm plastic container and were fed a diet of ground fishfood (TetraMin, Melle, Germany). All laboratory mosquitoes used in this study were 10 days post-ecdysis unless otherwise noted. For the experimental studies, mosquitoes were reared under three different environments: nondiapausing mosquitoes reared at 25°C in a long-day photoperiod (15 h:9 h, L:D), referred to as ND25; nondiapausing mosquitoes reared at 18°C in a longday photoperiod, referred to as ND18; and diapausing mosquitoes at 18° C in a short-day photoperiod (9 h:15 h, L:D), referred to as D18. Field-collected individuals were also used for one phase of this study. Females were obtained in the vicinity of Columbus, Ohio from August 2005–July 2006; males were not used because they do not survive the winter.

Relative humidities were generated by saturated salt solutions with excess solid salts: anhydrous CaSO₄ for 0% RH up to double-distilled water for 100% RH, within sealed glass or plastic desiccators (Winston and Bates, 1960; Johnson, 1940;

Toolson, 1978). The other salts used were: potassium acetate (23% RH), MgCl₂ (33% RH), NaNO₂ (65% RH), NaCl (75% RH), KCl (85% RH), KNO₃ (93% RH) and K₂SO₄ (98% RH). A hygrometer (s.d. \pm 0.7%RH; Taylor Scientific, Philadelphia, PA, USA) was used to monitor the relative humidity daily; readings varied less than 1% throughout the course of the experiment. Relative humidities in this experiment were expressed as water vapor activity (a_v =%RH/100) to allow comparison between the water activity (a_w) of the insect (0.99 a_w) and that of the surrounding test air (Wharton, 1985).

Mosquitoes used in the experiments were housed individually in a 20 ml mesh-covered chamber with no food or water and placed onto a perforated porcelain plate to prevent contact between the chamber and solutions at the bottom of the desiccators. An electrobalance was used for determining mass (precision, s.d. $\pm 0.2 \mu g$; accuracy, $\pm 6 \mu g$, at 1 mg; CAHN, Ventron Co., Cerritos, CA, USA). Individual mosquitoes were taken from the desiccator, removed from their enclosure, weighed and returned to their experimental conditions within 2 min. For a majority of the experiments, CO_2 anesthesia was used for immobilization. Results from this treatment were compared to smaller subsets that were exposed to -20° C for 2 min or had their wings clipped so they would not fly or received no treatments to ensure that the results of these experiments accurately represented the water balance profile of C. *pipiens*. So that all measurable mass changes reflected changes in body water levels rather than the effects of digestion, metabolism or excretion, the mosquitoes were held at 0.65 a_v , 25°C with no food until their mass had declined by 4–6% of the original body mass (Wharton, 1985; Benoit et al., 2005).

Water content

To determine water mass (M_w) inside the mosquito, dry mass (M_d) was subtracted from the initial mass (M_i) . To ensure that specimens were completely dry, the mosquitoes were killed in a frost-free freezer (-20°C, 6 h exposure) and placed at 70°C in 0.00 a_v . Mass values were taken daily until constant values were attained, indicating complete dryness. To relate the water content of mosquitoes to other arthropods, percentage body water content was determined according to Eqn 1 (Wharton, 1985):

 $M_{\rm w} = 100 \text{ x} (M_{\rm i} - M_{\rm d}) / M_{\rm i}$, (1)

The minimum amount of water that can be lost before irreversible dehydration was determined by exposing the mosquitoes to 0.33 and 0.93 *a*v at 25°C, and weighing them hourly until they lost the ability to right themselves. The mass at which individuals failed to respond to tactile stimulation is the critical activity point (CAP), and was used to calculate percentage change in mass:

 $M_{\rm w} = 100 \text{ x} (M_{\rm t} - M_0) / M_0$, (2)

where M_t is the mass at any time (*t*) and M_0 is the initial water mass. This point approximates the dehydration tolerance (Benoit et al., 2005).

Water loss

Based on standard water balance kinetics, if no water is available for uptake $(0.00 a_v)$ then changes in the water mass are solely from loss, with no interference from water molecules present in the environment or adhering to the insect cuticle (Wharton, 1985). To establish the water loss rates (transpiration=integumental plus respiratory water loss), mass values were taken hourly at 0.00 a_v , 25°C and displayed on a plot of ln (M_t/M_0) against time. Using Wharton's exponential model for water loss (Wharton, 1985) the slope (-kt) was expressed as a loss rate in % h⁻¹:

$$M_{\rm t} = M_0 \ge {\rm e}^{-k{\rm t}}$$
, (3)

To determine where water loss begins to increase rapidly, water loss measurements were determined for individual mosquitoes at multiple temperatures. If a point exists where water loss increases more rapidly, a critical transition temperature (CTT) is present. The CTT was established from Eqn 4:

$$\ln k = -Ea/(RT) + A$$
, (4)

where k is the water loss rate, E_a is the energy of activation, R is the gas constant, T is absolute temperature and A is the frequency factor. Live mosquitoes were used to allow for ecologically relevant comparisons (Benoit et al., 2005).

Water gain

To determine whether atmospheric water vapor was used as a source of water, the water mass of the mosquitoes was monitored at multiple water vapor activities (1.00, 0.98, 0.93, 0.85, 0.75, 0.65, 0.33 and 0.23 a_v). Water should be lost at all water vapor activities with diffusion promoting movement from the higher a_w (0.99 a_w) within the mosquito (Wharton, 1985) to the surrounding air with a lower a_v (>0.98 a_v). The lone exception is at saturation (1.00 a_v), where the gradient favors movement into the insect. Thus, if a mosquito can absorb water at a vapor activity below saturation, it is against the atmospheric gradient and indicates the presence of an active uptake mechanism. The lowest vapor activity where water loss can be countered by uptake from vapor in the air has been designated the critical equilibrium activity (CEA) (Wharton, 1985).

Uptake of free water was assessed by exposing mosquitoes to $50-60 \ \mu l$ droplets of deionized water stained with 0.1% Evans Blue dye. The drops were placed on a 100 mm x 15 mm Petri plate inside a 20 l chamber and 10 mosquitoes were allowed to freely approach the water. Observations were made at 3-h intervals with a dissection microscope at 40x magnification for a total of 15 h. After exposure, the mosquitoes were rinsed with deionized water and examined for the presence of blue coloration in the gut by dissection in 1.0% NaCl, using light microscopy at 100x magnification.

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Polyol and sugar accumulation

Glycerol content within the whole body of the mosquitoes was determined using a free glycerol assay (Sigma Chemical Co., FG0100) (Rivers and Denlinger, 1993; Yoder et al., 2006). First, groups of five adult mosquitoes were homogenized in 25 mmol I^{-1} sodium phosphate (pH 7.4) and centrifuged at 12 000 *g* for 10 min to remove insoluble insect debris. Deproteinization of the supernatant was accomplished by adding 6.0% perchloric acid and the precipitated protein was removed by centrifugation (12 000 *g* for 5 min). Samples were neutralized with 5 mol I^{-1} phosphate carbonate to pH 3.5. After addition of the glycerol reagent, concentrations were determined according to absorbance at 540 nm *versus* standard concentrations.

Sorbitol concentrations were determined (Bailey, 1959). Two mosquitoes were crushed in 1.0 ml of deionized water, and the mosquito debris was removed by centrifugation at 5000 *g* for 5 min. A portion (0.1 ml) of the supernatant was removed and combined with 0.2 ml of 0.3 mol l^{-1} barium hydroxide followed by 0.18 ml of zinc sulfate solution (5.0%, m/v with 0.004%, m/v Phenol Red). To remove excess barium hydroxide, 0.05 ml of magnesium sulfate solution (4.0%, m/v) was added. After centrifugation (12 000 *g* for 5 min), 1.0 ml of the supernatant was combined with 0.4 ml 1 mol l^{-1} sulfuric acid and 0.1 ml of 0.2 mol l^{-1} periodic acid. The reaction was allowed to proceed for 10 min and then arrested by the addition of 0.2 ml of 1 mol l^{-1} sodium arsenite solution. After 2 min, 1.0 ml of phenylhydrazine reagent (400 mg phenylhydrazine dissolved in 100 ml 0.42 mol l^{-1} HCl) along with 0.1 ml of 5% (m/v) potassium ferricyanide solution was added to start the colormetric

changes. The reaction was allow to proceed for 10 min and was followed by the addition of 2.3 ml of 4.2 mol l^{-1} hydrochloric acid to stabilize the magenta color. After 5 min the absorbance was measured at 540 nm and concentrations of sorbitol were determined by comparison to standard concentrations.

Trehalose and total sugar content were determined using a protocol similar to that of Van Handel (Van Handel, 1985a). Five adult mosquitoes were homogenized in 200 μ l sodium sulfate (2.0% w/v) at 25°C. The homogenate was combined with 1 ml methanol and centrifuged at 12,000 g for 2 min. The supernatant was removed, and the previous step was repeated with 0.5 ml methanol to ensure that all the trehalose was in the supernatant. Samples were concentrated to 0.5 ml. The volume representing the cuticular lipid content for one mosquito (0.1 ml) was combined with 1 mol l^{-1} HCl in a 16 mm x 100 mm tube and heated at 90°C for 7 min. Immediately after heating, 0.15 ml NaOH was added and the samples were again heated to 90°C for 7 min. Anthrone reagent (150 ml distilled water, 380 ml concentrated sulfuric acid, 750 mg anthrone) was added to 5 ml and the sample was heated to 90°C for 17 min. Once cooled to room temperature the absorbance was measured at 625 nm to find the concentration of trehalose. For determination of total sugar content, individual mosquitoes were crushed in 5 ml anthrone reagent, heated for 17 min at 90°C and the optical density was measured at 625 nm. Both trehalose and total sugar concentrations were established by comparison to the absorbance of standards.

Cuticular lipid quantification

Cuticular lipids were quantified by analyzing groups of females reared under the three developmental regimens (ND25, ND18 and D18) (Yoder et al., 1992). First, the hydrocarbons (and other nonpolar surface lipids) were removed from the external surface of the mosquito by washing the groups (N=30) with chloroform:methanol (2:1, v/v) three times for 5 min. After the extracts were concentrated to dryness with N₂, each sample was redissolved in 200 µl chloroform:methanol. This extract was passed through a silica gel column (Millipore, Billerica, MA, USA) to elute hydrocarbons (and other nonpolar lipids) with hexane (2.0 ml) and polar lipids with chloroform (2.0 ml). Samples were dried on predesiccated (0.00 a_v , 25°C for 5 days) aluminum pans using a constant flow of N₂. The lipids on each pan were weighed after 48 h, and then reweighed at 96 h and 120 h to verify complete dryness. Samples were collected immediately after emergence and every subsequent fifth day until 80 days.

Fat reserve assay

The rate at which the female mosquitoes utilized their fat reserves during starvation was calculated as an indirect index of metabolic rate. Mosquitoes were held at 0.85 a_v , 18°C with water *ad libitum* but without access to food. Starvation was initiated 10 days after adult emergence. The overall lipid content of the mosquitoes was measured prior to starvation and every subsequent fifth day for 30 days. Total lipid content was analyzed (Van Handel, 1985b). Briefly, the lipids from individual mosquitoes were extracted in 0.5 ml chloroform:methanol (2:1). The sample was centrifuged (2500 g for 5 min) to remove insoluble debris, and solvent was evaporated by heating (90°C). The remaining lipids were dissolved in 0.2 ml sulfuric

acid and transferred to a 10 ml test tube. Vanillin reagent (600 mg vanillin, 100 ml water, 400 ml 85% phosphoric acid) was combined with the lipid solution to bring the volume to 5 ml. After 10 min the absorbance was measured at 520 nm and compared to standard solutions to determine the lipid content.

Sample size and statistics

For water balance experiments, each measurement was replicated three times with 20 mosquitoes per replicate. Means for the polyol and lipid experiments were based on 10 replicates of five individuals. Calculated means \pm s.e.m. were compared using one- and two-way analysis of variance (ANOVA) with arcsin transformation in the case of percentages. Data derived from regression lines were assessed by testing for the equality of slopes (Sokal and Rohlf, 1981).

Results

Water pool

Overall water balance profiles of pupae and adult males and females at 10 days post-emergence are shown in Table 3.1. In all cases, dry mass correlated positively with water mass (r^2 =0.94), indicating that water flux was standardized according to size (Wharton, 1985). Pupae and males retained the same water mass, dry mass and water content at all temperatures and photoperiods tested (Table 3.1; ANOVA, *P*>0.05), but the levels observed in these two developmental stages were significantly different (ANOVA, *P*<0.05). By contrast, the water content of females did not remain constant. Under diapause-inducing conditions (D18), the initial mass was significantly higher (Table 3.1; ANOVA, P < 0.05) than in those reared under nondiapausing conditions at the same (ND18) or higher temperature (ND25). This was the result of a significant increase in the dry mass of diapausing mosquitoes that was not accompanied by an increase in water mass. Since the dry mass increased and the water mass remained constant, the percentage water content in diapausing females declined 12–13% in relation to nondiapausing mosquitoes. Only the size of adult females was affected by diapause conditions, a result consistent with previous observations of this mosquito (Buxton, 1935; Spielman and Wong, 1973; Robich and Denlinger, 2005).

Water loss

With no water available for uptake at 0.00 a_v , water loss can be analyzed with no interference by external water vapor (Wharton, 1985; Benoit et al., 2005). This allows water loss to occur as an exponential decay in a first-order kinetic relationship that can be analyzed according to Wharton (Wharton, 1985). As with the overall water pool, temperature and photoperiod had little effect on water loss rates in males (5% h⁻¹) and pupae (7% h⁻¹) (Table 3.1). Additionally, pretreatments of -20°C for 2 min, CO₂ knockdown and clipping of wings had no effect on the water loss rates (data not shown). Nondiapausing females (ND18 and ND25) lost water at a rate of 3.5% h⁻¹, a rate significantly more rapid than in diapausing individuals (D18) that lost water at 2.3% h⁻¹ (Fig. 3.1). As long as the female mosquitoes were held under diapause-inducing conditions, their water loss was depressed, but when diapause was broken, the rate of water loss increased until it was comparable to that of nondiapausing individuals (Fig. 2.2). Temperature increases had nearly identical effects on the water loss rates of diapausing and nondiapausing individuals, based on the CTT values observed (Table 3.1). Pupae had the lowest CTT at 35–36°C, followed by males (38–39°C) and then females (40–42°C).

Dehydration tolerance

Nondiapausing adult females survived for 10–12 h at 0.00 a_v , 25°C, whereas diapausing females lived for 18–20 h under these conditions. Interestingly the dehydration tolerances for ND25, ND18 and D18 mosquitoes were nearly identical; all females were capable of losing 40% of their water before they succumbed to desiccation (Table 3.1). Based on the water loss rate of 2.3% h⁻¹ for the diapausing females and their dehydration tolerance of 40%, these mosquitoes should survive for approximately 20 h, a value identical to their measured survival time of 18–22 h. The water loss and dehydration tolerance of males and pupae also correlated with their survival time. Overall, diapause has no effect on the level of dehydration that *C. pipiens* can tolerate.

Water uptake

When water content was monitored at water activities below saturation $(<1.00 a_v)$, absorption of water vapor was not sufficient to counter loss in any developmental stage tested (Fig. 3.3). Water was lost at all activities below saturation, thus placing the CEA at >0.99 a_v , which is the only point where water uptake can occur. In all cases, water loss decreased with increasing a_v (r^2 >0.98; ANOVA,

P < 0.005, when analyzed without 1.00 a_v) indicating that passive gain of water may occur by chemisorption of water to the mosquito cuticle, but this water can only reduce, not completely counter, water loss. Water loss should decrease linearly until it is zero at 1.00 a_v for insects that cannot absorb water vapor (Hadley, 1994), but, interestingly, this was not the case: water loss increased rapidly between 1.00 a_v and 0.98 a_v (Fig. 3.3) and thereafter the relationship was linear. To further verify that water vapor could not be utilized, mosquitoes were first desiccated at 0.85 a_v until a loss of 15% of the water mass occurred, transferred to 0.98 a_v (the highest water activity where water loss should occur passively) and then monitored for mass change. In all cases, the water mass continually declined when the mosquitoes were moved from 0.85 a_v to 0.98 a_v (data not shown), indicating that even predesiccation did not prompt water vapor uptake. For *C. pipiens*, water vapor is not a primary source for replenishing internal water pools.

The possibility of water gain from metabolism was also tested. The production of metabolic water should reduce dry mass of the mosquitoes if individuals are exposed to dehydrating conditions and then allowed to rehydrate (Yoder and Denlinger, 1991a). Using a hydration (1.00 a_v until constant mass):dehydration (0.75 a_v for 2 days) comparison to a hydration (1.00 a_v until constant mass):dehydration (0.75 a_v for 2 days):rehydration (1.00 a_v until constant mass) regimen, followed immediately by drying (90°C until constant mass), revealed no differences in dry mass for the ND25, ND18 or D18 mosquitoes. This suggests that water from metabolism is not responsible for the large portion of water gained by nondiapausing or diapausing mosquitoes. In the presence of Evans Blue-stained droplets, mosquitoes that had been dehydrated made deliberate movements toward the water. Mosquitoes near the water droplets would walk to the edge and insert their proboscis into the dyed fluid. As the mosquito drank the stained droplets its gut acquired a blue coloration that was noticeable without magnification. Once the mosquito had removed its proboscis from the droplet, uptake was verified by the presence of blue dye in the gut (40x magnification, 0.1% saline dissection). All three experimental groups of adult mosquitoes (ND25, ND18, D18) were capable of liquid water uptake in this manner. For *C. pipiens*, liquid water and blood feeding are the primary sources of water replenishment.

Water requirements of field-collected mosquitoes

Field-collected mosquitoes obtained between September 2005 and March 2006 are referred to as winter-acclimated mosquitoes and those from April to August 2006 are defined as summer-acclimated. The dry and initial masses of winter-acclimated individuals were higher than those adapted for summer (Fig. 3.4). The increase in dry mass, as in the laboratory experiments, resulted in lower percentage water content (data not shown). Water loss rates were highest in summer-acclimated female mosquitoes, and declined in September by 30%; water loss increased only slightly throughout the fall and winter (Fig. 3.4). The CTT was the same for winter-and summer-acclimated mosquitoes ($40.2 \pm 0.9^{\circ}$ C). Like the lab-reared mosquitoes, there was no point at which water could be absorbed from subsaturated air, and internal water was replenished solely by liquid water uptake and blood feeding.

Overall, the water requirements of winter- and summer-acclimated individuals were similar to those of diapausing and nondiapausing individuals, respectively.

Polyol content

Glycerol, sorbitol, trehalose and total sugar contents of ND25, ND18 and D18 mosquitoes are presented in Fig. 3.5. Glycerol and sorbitol concentrations were not significantly different among the three experimental groups, nor did concentrations change much with age (ANOVA, P>0.05; Fig. 3.5A,B). Trehalose concentrations were significantly higher for the diapausing groups (D18) between 5 and 30 days, but after 30 days no significant differences were noted among the ND25, ND18 and D18 groups (ANOVA, P>0.05). Like trehalose, total sugar content was elevated in early diapause, but decreased below that of nondiapausing mosquitoes after 55 days. In the field-collected mosquitoes, no significant differences were noted for glycerol, sorbitol or trehalose in samples collected in November 2005, February 2006 and June 2006 (Table 3.2). We thus conclude that these polyols have little effect on the ability of *C. pipiens* to retain water.

Cuticular lipids

Females of *C. pipiens* that were in diapause had more than twice as much cuticular hydrocarbons as individuals reared under nondiapausing conditions at both 18 and 25°C (Fig. 3.6). When diapausing females were transferred to long-day conditions to break diapause, the amount of cuticular lipids declined, but within the timeframe of our experiments the low levels observed in nondiapausing individuals were never reached (ANOVA, P < 0.05). Potentially, the larger size of the diapausing females could account for the observed increase of cuticular lipids, but this was not the case as indicated by calculations based on a per mg basis. Diapausing females contained 93.2 ± 6.2 ng of hydrocarbons per mg of mosquito, whereas nondiapausing females contained 63.4±9.4 ng mg⁻¹, thus the cuticular hydrocarbon content is higher in diapausing mosquitoes on a per mg basis. Unlike nonpolar cuticular hydrocarbons, cuticular polar lipids showed no differences between individuals reared at ND25 (1.42 ± 0.22 µg/mosquito), ND18 (1.31±0.31 µg/mosquito), and D18 (1.34 ± 0.31 µg/mosquito). For field-collected mosquitoes, only individuals obtained from November 2005 and June 2006 were analyzed. Individuals collected during the winter had more hydrocarbons (390 ng/mosquito) than those collected during the summer (187 ng/mosquito) (ANOVA, *P*<0.05), and no difference occurred in the polar lipids (ANOVA, *P*>0.05). Thus, diapausing mosquitoes consistently have more cuticular hydrocarbons, and these may be key to reducing water loss.

Fat utilization

Nondiapausing mosquitoes, reared at 18 or 25°C, utilized lipids much faster than diapausing mosquitoes (Fig. 3.7). Temperature had a significant effect (ANOVA, P < 0.05) on how quickly fat reserves were utilized, with the rate nearly twice as high for nondiapausing mosquitoes at 25°C (1.31% day⁻¹) than at 18°C (0.68% day⁻¹). Additionally, it is important to note that this same relationship persisted when absolute values (mg day–1), rather than proportional values (% day–1), were used. For example, over a 30 day period mosquitoes at D18 lost 0.12 ± 0.02 mg lipids and those at ND18 lost 0.17 ± 0.02 mg lipids, which is a significant difference (ANOVA, *P*<0.05). Overall, diapausing mosquitoes used lipids at the slowest pace (0.24% day–1), a feature the presumably correlates with the reduced metabolism of diapausing mosquitoes.

Discussion

Water balance of nondiapausing mosquitoes

The tolerance for water loss has been thoroughly investigated in a wide range of arthropods. Most insects are capable of tolerating a 20–30% reduction in their internal water pool before succumbing to desiccation, and a few can tolerate losses exceeding 70% (Hadley, 1994; Suemoto et al., 2004). The dehydration tolerance of males (38%) and females (40%) of C. pipiens that we observed in this study was higher than most insects, including other mosquitoes, e.g. Anopheles arabiensis (29%) and A. gambiae (33%) (Gray and Bradley, 2005). The 65% water content for C. pipiens females and 70% for males falls within the 65–75% water content that is common for flies (Arlian and Eckstrand, 1975; Hadley, 1994) and is similar to the 70–75% content reported in other mosquitoes (Gray and Bradley, 2005). This is the first report on the water content and dehydration tolerance of mosquito pupae. Their 78% water content is much higher than the pupae of two other dipterans, Sarcophaga bullata (65%) (Yoder and Denlinger, 1991a) and Peckia abnormis (67%) (Yoder and Denlinger, 1991b). The point of dehydration at which C. pipiens pupae failed to eclose, approximately a 30% loss, was also high in comparison to the 25% reduction in water content observed in other fly pupae (Yoder and Denlinger, 1991b).

To maintain water levels, insects commonly imbibe liquid water or obtain water from their food. Many dipterans are known to drink liquid water (Hadley, 1994), and the adults of C. *pipiens* are no exception. At no point in this study was there evidence that this mosquito could balance water loss with gain from subsaturated air. Water was lost at all subsaturated relative humidities as a result of simple diffusion. This was experimentally verified by the higher daily losses in water observed at lower water vapor activities. But at 1.00 a_v , water gain was observed because at that point the water content of the air was higher than that of the mosquito $(0.99 a_w)$. A lack of water vapor absorption is fairly common, and indicates a CEA>0.99 a_v , and thus water must be acquired from liquid or food intake (Arlian and Ekstrand, 1975; Hadley, 1994). The importance of water uptake was verified in this study by direct observation of liquid water uptake, and in a previous study (Rinehart et al., 2006) by the failure of this mosquito to survive for prolonged periods if no free water was present. Of interest is the rapid increase of water loss that occurs when mosquitoes are held at 0.98 a_v when compared to 1.00 a_v . Although water loss linearly increases from 0.99 to 0.00 a_v , the large increase between 1.00 and 0.98 a_v suggests a different relationship operating at higher relative humidities. One possible explanation for this is that C. pipiens is much more active when vapor activities are high, thus increasing the rate of water loss from respiration under these conditions. Then, below 0.98 $a_{\rm v}$ the respiration rate possibly decreases and thereafter remains constant, allowing water loss to increase linearly with further decreases in vapor activity.

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Water loss rates are the best indicators for assessing the suitability of an insect for a particular habitat. In most cases, suppressed water loss rates indicate that the species is adapted for a dry environment, and rapid loss rates suggest a preference for more humid environments (Wharton, 1985; Hadley, 1994; Benoit et al., 2005). Since water loss rates have been determined for the adults of only three mosquito species, establishing a strong correlation between water loss and habitat preference is premature, but Gray and Bradley determined the water loss rates of adult females for both A. arabiensis and A. gambiae to be 57 μ g h⁻¹ (Gray and Bradley, 2005), which converts to 7.0% h^{-1} and 7.5% h^{-1} when analyzed in relation to their overall water content and size, and interestingly, both of these sub-Saharan mosquitoes reside in a desiccation prone area where we might anticipate that water loss rate would be reduced. But this is not the case; the rates of water loss in C. pipiens were nearly 50% lower than observed with the two Anopheles species. Possibly this is due to the fact that C. pipiens is active during the dry summer months in temperate zones, whereas the Anopheles sp. are most abundant during the rainy seasons in Africa. C. pipiens is also nearly twice the size of A. gambiae and A. arabiensis, thus reducing the surface area to volume ratio.

The CTT represents a transition temperature at which water loss begins to increase rapidly. Though the CTT was previously thought to represent a change in phase of cuticular lipids, this interpretation has recently been questioned (Yoder et al., 2005). Even so, the CTT of *C. pipiens*, 40°C, is toward the upper end of CTTs commonly reported for other insects, 30–45°C (Hadley, 1994). This suggests that
both males and females of *C. pipiens* are fairly tolerant of water loss at high temperatures.

Comparison with diapausing mosquitoes

The water balance characteristics of diapausing females were very different from those observed in nondiapausing females, but males, which do not enter diapause (Spielman and Wong, 1973), showed no differences when reared under environmental conditions that produced diapause in females. No differences in water requirements were noted in pupae under the two conditions, thus the distinction between diapause and nondiapause water balance characteristics is only evident after adult eclosion. This is consistent with the observation that specific molecular changes induced by diapause in *C. pipiens* do not occur until at least 1 day after adult eclosion (Robich and Denlinger, 2005).

Diapausing females are much larger than their nondiapausing counterparts, as indicated by a twofold increase in dry mass. This large increase in dry mass resulted in a significant reduction in the percentage water content of the diapausing females when compared to nondiapausing females. Water content (53%) was particularly low, a feature commonly associated with insects that are more resistant to dehydration (Hadley, 1994; Benoit et al., 2005). Low body water content is usually associated with high amounts of stored lipids and/or heavily waterproofed cuticle. The large increase of dry mass observed in *C. pipiens* is presumably a consequence of the upregulation of lipid metabolism that has been documented both physiologically

(Buxton, 1935; Mitchell and Briegel, 1989) and at the molecular level (Robich and Denlinger, 2005).

With water loss rates suppressed by 30%, it is apparent that diapausing mosquitoes are more tolerant of desiccation than their nondiapausing counterparts. Although both diapausing and nondiapausing females can survive a loss of approximately 40% of their body water, the diapausing females are able to survive 20 h when exposed to 0.00 a_v compared to only 12 h for nondiapausing females. This survival time is significantly less than reported for the same strain (Rinehart et al., 2006), but this discrepancy can readily be explained by the fact that experiments described here were conducted at 25°C rather than 18°C, and the mosquitoes used here were analyzed individually rather than in groups, as previously described (Rinehart et al., 2006). Diapausing and nondiapausing mosquitoes responded similarly to changes in temperature, as indicated by the two groups having nearly identical CTTs.

Several features are likely to work concurrently to reduce water loss during diapause. A reduction in the rate of metabolism that is associated with diapause (Denlinger, 2002) is probably a key factor, and the suppressed oxidation of lipids in diapausing mosquitoes observed in this study suggest that less water was lost from respiration. The greater body size of diapausing females lowers the surface area to volume ratio, and in turn, proportionally fewer water molecules are lost. The potential contribution of cuticular lipids was also tested in this study, and our results suggest that the production of extra cuticular hydrocarbons contribute to the reduced water loss observed in diapausing females. The deposition of additional cuticular lipids is a

common mechanism for suppressing water loss in a variety of insects and other arthropods (Toolson, 1982; Hadley, 1994). Qualitative differences in the cuticular lipids were not tested, but based on previous studies on the lipid content of mosquitoes, it is unlikely a significant change in major constituents occurs (Van Handel, 1967).

It is not probable that the three polyols that we tested (glycerol, sorbitol and trehalose) contributed to the reduction of water loss during diapause, as known in other species (Yoder et al., 2006). We also detected no large differences in the overall sugar content. The high content of trehalose we observed is similar to the level noted in C. pipiens fatigans (Lakshmi and Subrahmanvam, 1975) and may contribute to the relatively high dehydration tolerance we observed for C. pipiens but not to differences in the water loss rates. The initial increase of trehalose and the overall sugar content is probably due to the increase of sugar uptake used to generate lipids in diapausing mosquitoes immediately after adult emergence (Lakshmi and Subrahmanvam, 1975; Robich and Denlinger, 2005) and is not an adaptation to reduce stress. Overall, polyols and related compounds are not likely candidate molecules contributing to the enhanced desiccation tolerance observed during diapause in this species. The lower water loss rates of diapausing C. pipiens is most likely a consequence of their larger size, reduced metabolic rate and the production of additional cuticular hydrocarbons.

Comparison with field-collected mosquitoes

Mosquitoes collected from the field displayed nearly identical water balance profiles as observed under laboratory conditions. During the spring and summer months, the water balance characteristics closely resembled the features of nondiapausing mosquitoes reared in the laboratory, whereas mosquitoes collected in the fall and winter displayed moisture requirements nearly identical to laboratory mosquitoes reared under diapausing conditions. Additionally, the amount of cuticular hydrocarbon was higher in overwintering individuals than in those collected during the summer, but like the nondiapausing and diapausing females, no differences were noted in the polar lipids or polyols. The only difference we noted between the field and laboratory mosquitoes was that the field-collected mosquitoes were much smaller than the mosquitoes reared in the laboratory, the likely consequence of suboptimal conditions in the wild during larval development. Reducing the feeding of laboratoryreared mosquitoes by 40–50% reduced mosquitoes to a size similar to the fieldcollected mosquitoes (J. B. Benoit, personal observation), but the percentage water content was not different between the two groups, thus indicating that the fieldcollected mosquitoes were only smaller than ones reared in the laboratory. Water loss rates were also higher for the summer field-collected mosquitoes in comparison to the laboratory-reared mosquitoes, a feature that we attribute to the size differences. Though the baseline water loss rates in field-collected and nondiapausing individuals differed, the water loss rates for those entering winter in the field were similar to those entering diapause in the laboratory, and the same mechanisms appear to be used to suppress water loss, thus we feel confident that our laboratory observations are a valid reflection of the physiological responses operating in the field.

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		Pupae			Males			Females	
Characteristic	ND25	ND18	D18	ND25	ND18	D18	ND25	ND18	D18
Water content									
Initial mass (mg)	3.45±0.09	3.47±0.08	3.59±0.11	2.01±0.10	2.10 ± 0.20	2.15±0.15	3.60±0.10	3.66±0.12	5.16 ± 0.21^{a}
Dry mass (mg)	0.76±0.08	0.72±0.09	0.82±0.09	0.58±0.09	0.63 ± 0.09	0.63±0.16	1.20 ± 0.11	1.26 ± 0.15	2.42 ± 0.19^{a}
Water mass (mg)	2.69 ± 0.07	2.75 ± 0.07	2.77 ± 0.08	1.43 ± 0.11	1.47 ± 0.11	1.52 ± 0.19	2.40 ± 0.13	2.40 ± 0.16	2.74 ± 0.18
Water content (%)	77.9±1.4	79.2±1.6	77.1±1.1	71.2±1.8	70.8±1.3	70.8±1.2	66.7±1.3	65.5 ±1.4	53.1±1.2 ^a
Water loss									
Rate (% h ⁻¹)	7.17±0.16	7.26±0.11	7.08±0.13	5.08±0.09	4.80 ± 0.10	4.71±0.15	3.57 ±0.14	3.37±0.15	2.31 ± 0.16^{a}
Loss tolerance (%)	29.2±0.9	28.9±1.1	30.1±1.4	33.1±1.0	33.3±1.3	33.9±1.1	35.4 ±0.9	36.3 ±1.2	35.5±1.7
CTT (°C)	36.2±2.1	35.3±1.7	36.3±1.3	39.4±2.1	38.3±2.8	38.3±1.9	41.2±1.8	40.3 ±1.9	41.4±1.8
Water gain									
Free water uptake	NA	NA	NA	+	+	+	+	+	+
$CEA(a_v)$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C; CTT, critical transition temperature; CEA, critical equilibrium humidity; +, does occur; NA, not applicable.

Superscript letters indicate that a value is significantly different for environmental condition within a particular stage or sex. Values are means ± s.e.m. of 60 individuals.

Table 3.1. Comparison of the water requirements of pupae, males and females of *Culex pipiens* reared under diapausing and nondiapausing conditions.

		Date of collection	n	
Concentration	Nov. 2005	Feb. 2006	July 2006	
Polyols (ng g ⁻¹ mosq	uito)			
Glycerol	52.1±6.3	49.3±9.2	56.5±6.9	
Sorbitol	31.5±5.2	28.9±0.6	36.6±5.2	
Sugar (mg g ⁻¹ mosqu	uito)			
Trehalose	1.21±0.15	0.96 ± 0.10	1.22±0.16	
Total sugar	5.34 ± 0.29	4.72±0.31	4.83±0.41	
Cuticular lipids (ng/n	nosquito)			
Hydrocarbons	440±65	412±62	220 ± 42^{a}	
Polar components	1310±120	1160 ± 215	1310±192	

Values are means \pm s.e.m. of 10 determinations. A superscript letter indicates the value is significantly different than others in the row.

Table 3.2. Amounts of polyols, sugars and cuticular lipids in field-collected populations of *Culex pipiens* collected from Columbus, Ohio, USA.



Fig. 3.1. Net water loss rates in females of *Culex pipiens* reared under diapausing (D18) and nondiapausing conditions (ND25; ND18) at 0.00 a_v . The slope of the regression through the points represents the water loss in % h⁻¹. M_t is the mass at any time *t* and M_0 is the initial water mass. Values are means ± s.e.m. of 60 mosquitoes. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C.



Fig. 3.2. Water loss rates in females of *Culex pipiens* over a prolonged period. Each point represents water loss determined as in Fig 3.1. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C. D18 to ND25 at 40 days indicates the mosquitoes were moved from diapausing (D18) to nondiapausing (ND25) conditions after 40 days, to break diapause.



Fig. 3.3. Water vapor exchange at different vapor activities in females of *Culex pipiens*. For each point, the vapor exchange was determined as in Fig. 3.1. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C.



Fig. 3.4. Water balance characteristics of *Culex pipiens* females collected at monthly intervals from the field in Columbus, Ohio. WLR, water loss rate (% h^{-1}); M_d , dry mass (mg); M_w , water mass (mg); M_i , initial mass (mg). Values are means \pm s.e.m. of 30 mosquitoes.



Fig. 3.5. Polyol and sugar content of nondiapausing and diapausing adult females of *Culex pipiens*. (A) Sorbitol; (B) glycerol; (C) trehalose; (D) total sugars. Closed squares, ND25 mosquitoes, reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; open squares, ND18 mosquitoes, reared under a nondiapausing photoperiod at 18°C; closed diamonds, D18 mosquitoes, reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C; open diamonds, D18 to ND25 at 40 days. Values are means \pm s.e.m. of 10 replicates of five individuals each. All error bars are smaller than the symbols.



Fig. 3.6. Amount of cuticular hydrocarbons extracted from nondiapausing and diapausing females of *Culex pipiens*. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C; D18 to ND25 at 40 days, indicates the mosquitoes were moved from diapausing (D18) to nondiapausing (ND25) conditions after 40 days, to break diapause. Values are means \pm s.e.m. of 10 replicates of five mosquitoes each.



Fig. 3.7. Proportional reduction of internal lipid reserves in nondiapausing and diapausing females of *Culex pipiens*. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C. Values are means \pm s.e.m. of 10 replicates of five mosquitoes each.

Chapter 4: Repeated bouts of dehydration deplete nutrient reserves and reduce egg production in the mosquito, *Culex pipiens*

Abstract

In this study of the mosquito, *Culex pipiens*, we examined the impact of multiple bouts of dehydration and rehydration on survival, depletion of metabolic reserves and egg production in both nondiapausing and diapausing females. Mosquitoes provided access to sugar during rehydration survived longer than those allowed to rehydrate without sugar, and their survival was similar to that of mosquitoes of the same age that were not dehydrated. Among mosquitoes not provided sugar, each dehydration bout reduced the mosquito's dry mass, an effect likely due to the utilization of carbohydrates and lipid reserves. The toll on glycogen and lipid reserves is likely to be especially costly for diapausing mosquitoes that are dependent on these stored reserves for winter survival. Egg production of both nondiapausing and post-diapause C. pipiens was also reduced in response to multiple bouts of dehydration. Although egg quality was not compromised, the number of egg produced was reduced. Both nondiapausing and diapausing females can compensate for the nutrient loss due to dehydration by sugar feeding, but the opportunity to feed on sugar is likely to be rarely available in the overwintering habitat of diapausing females, thus the impact of dehydration may be especially pronounced in overwintering populations of C. pipiens.

Introduction

The response of mosquitoes to dehydration, as with other insects, is defined by their ability to maintain the water pool necessary to remain functional and to prevent or recover from the stress of dehydration (Hadley, 1994). Water balance is maintained by reducing water lost through cuticular and respiratory routes, improving water reabsorption by the alimentary canal, or increasing water uptake by drinking or absorbing water vapor. Water stress is alleviated by increasing the internal concentrations of protective sugars and polyols and by up-regulating expression of stress-related proteins that repair damaged proteins, reduce oxidative stress and maintain cellular integrity (França et al., 2007; Li et al., 2009; Lopez-Martinez et al., 2009). Previous studies have usually focused on a single bout of dehydration and rehydration to establish an insect's water balance profile (Wharton, 1985; Hadley, 1994; Benoit, 2009), but insects are likely to experience multiple bouts of dehydration, especially overwintering insects that remain dormant (in diapause) for many months.

Based on several studies of mosquito dehydration (Gray and Bradley, 2005; Benoit and Denlinger, 2007; Rinehart et al., 2006; Benoit et al., 2009; Lee et al., 2009), it is evident that mosquitoes are fairly susceptible to dehydration; i.e. they are hydrophilic. Adult female mosquitoes contain 60-70% water, and they can lose approximately 25-35% of their water content before succumbing (Benoit et al., 2009). To increase their water pool, mosquitoes rely solely on ingestion of blood or free water (Gray and Bradley, 2005; Benoit et al., 2009). Upregulation of heat shock proteins (Hsp70 and Hsp90) has been noted in three mosquito species, and knock-down experiments using RNA interference indicate that these two proteins are important for maintenance of dehydration tolerance (Benoit et al., 2009). Additionally, the diapause program increases resistance of adult females of *C. pipiens* to dehydration (Rinehart et al., 2006; Benoit and Denlinger, 2007), and differences in dehydration resistance have also been noted between M and S forms of *Anopheles gambiae* (Lee et al., 2009), thus both developmental programs (diapause and nondiapause) and population differences are known to impact a mosquito's dehydration resistance.

In this study, we examined the effect of multiple dehydration-rehydration exposures on the physiology of the northern house mosquito, *Culex pipiens*. To do so, we analyzed the water balance characteristics and energy reserves of mosquitoes exposed to single and multiple bouts of dehydration and compared the results with individuals of the same age held under non-desiccating conditions. Additionally, we provided a subsample of mosquitoes with sugar, and others were provided no sugar between dehydration bouts to determine whether the negative consequences of dehydration bouts could be alleviated by the presence of a nutrient resource. Finally, we compared effects of multiple dehydration bouts on both nondiapausing and diapausing adult females. In response to short daylength and low temperatures in late summer and autumn, adult females enter an overwintering diapause. Blood feeding ceases, females seek sugar resources to increase their fat reserves, and reproduction is halted (Spielman and Wong, 1973; Bowen et al., 1988). Diapause represents an extended period when food is not normally available, thus the effects of dehydration bouts could be expected to be particularly stressful at this time. We demonstrate that multiple bouts of dehydration/rehydration, in the absence of sugar, significantly impact survival of *C*. *pipiens*, most likely a result of depleted nutrient reserves. The consequences are also evident as a decrease in the number, but not quality, of eggs produced.

Materials and Methods

Mosquitoes

Culex pipiens (Buckeye strain) was reared as previously described (Robich and Denlinger, 2005). Larvae were fed a diet of ground fish food (Tetramin, Tetra, Mulle, Germany). Nondiapausing (ND) adult females were generated by rearing larvae and adults under long day conditions (15:9, L:D), while diapausing (D) females were reared under short day conditions (9:15, L:D). Both ND and D mosquitoes were reared at 18°C, 93% RH. Adults were fed 10% sucrose. Eggs were generated by allowing mosquitoes to feed on a live rooster (*Gallus gallus*; IACUC 2008A0206).

Dehydration experiments

Adult females 7 d post-emergence were used in this study. During each period of dehydration, mosquitoes were held at 0% RH until individuals lost 25% of their water content, moved to 100% RH in the presence of liquid water to rehydrate, and then moved to colony conditions (93% RH in the presence of free water until the next period of dehydration). A diagram of the dehydration bouts is presented as Fig. 1. During rehydration, one subset of mosquitoes was provided only water (Water-only) and a second group was provided 10% sucrose and water (Sugar + Water), thus providing a

comparison of mosquitoes exposed to dehydration/rehydration bouts with and without an energy source. Additionally, a group of mosquitoes was held at 100% RH throughout the experiments (Fully-hydrated) to ensure that differences were not due to age. This group served as a control, providing a comparison to mosquitoes that did not experience dehydration. In this study, nondiapausing mosquitoes were compared after 0, 1, 2, 4, 6, 8, and 10 bouts of dehydration, and diapausing mosquitoes were analyzed after 0, 5, 10, 15, 20, and 25 bouts of dehydration.

Water balance analysis

Mass changes in the mosquitoes were monitored gravimetrically using an electrobalance (CAHN 35, Ventron Co., Cerritos, CA). Each mosquito was weighed singly without enclosure after a brief CO₂ knockdown according to Benoit and Denlinger (2007). Mosquitoes were transferred to the weighing pan and returned to the experimental conditions within 1 min. Relative humidities were generated using saturated salt solutions (Winston and Bates, 1960), 0% RH was established with calcium sulfate, and double-distilled water was used to create 100% RH. All test relative humidities were validated with a hygrometer (Taylor Scientific, St. Louis, MO). All observations were conducted at 18°C in a controlled environment room.

The amount of water available for exchange (m, water mass) was determined according to standard methods for insects (Wharton, 1985; Hadley, 1994; Benoit and Denlinger, 2007). Mosquitoes were placed at 0%, 18°C until a loss of 4-6% of their mass to ensure that mass changes reflected a shift in the water pool (Wharton, 1985). Subsequently, consecutive mass determinations (0% RH, 22-24°C) were made at hourly intervals for a total of six mass readings, and then individuals were transferred to 80°C, 0% RH to determined dry mass (d, denoted by five consecutive daily mass measurements with no changes). Water mass was determined by subtracting dry mass from the initial mass. Initial, immediate and final water mass values were analyzed according by Wharton (1985) for determining water loss rates:

 $m_t = m_0 e^{-kt}$

where m_t is the water mass at any time t, m_0 is the initial water mass, and k is the rate of water loss expressed as %/h. The dehydration level at which the mosquitoes can no longer right themselves and fly when prodded was defined as the critical activity point. This denotes the dehydration tolerance limit, an irreversible lethal amount of water loss.

Nutritional reserve analyses

The amount of sugar, glycogen and lipids within each mosquito was determined using anthrone and vanillin assays (Van Handel, 1985a, 1985b, Van Handel and Day, 1988; Vaidyanathan et al., 2008). Briefly, individual mosquitoes were homogenized in 0.2 ml sodium sulfate, followed by the addition of 1.8ml of chloroform:methanol. The supernatant was divided equally into two 16 x 100 mm glass test tubes for determination of lipid and sugar content according to Van Handel and Day (1988). The precipitate was utilized to determine glycogen levels. Each glycogen sample was heated with 1 ml of anthrone reagent for 10 minutes, and then combined with 4 ml of hot anthrone reagent for 10 minutes. All samples (glycogen, lipid and sugar) were cooled to room temperature and absorbance was measured with a spectrophotometer at two wavelengths (625 and 560 for sugar and glycogen samples; 525 and 490 for lipid samples). Two samples were measured for each mosquito. Concentrations were determined using a standard curve.

Total protein content in the mosquitoes was determined with a Bradford Assay (Bio-Rad, Hercules, CA). Individual mosquitoes were placed in 500µl of phosphate buffered saline (PBS) and sonicated. The homogenate (100µl) was combined with 700µl PBS and 200µl Bradford reagent. Samples were incubated 10 min at 25°C and absorbance at 295 nm was determined. Known amounts of bovine serum albumin were used to establish a standard curve.

Egg production

To determine egg production, mosquitoes that had been exposed to multiple bouts of dehydration were subsequently allowed to bloodfeed on a rooster (*G. gallus*). Nondiapausing mosquitoes were exposed to their respective number of dehydration bouts, then held at 100% RH until they were 18d post adult emergence. Diapausing mosquitoes were held under diapausing conditions for 50d, then moved to nondiapausing conditions for 10 d before being offered a blood meal. After three days, a 1 liter container filled with 0.5 l water was provided for oviposition. The number of eggs in each raft was counted, and carbohydrate, glycogen and lipid levels within the eggs were analyzed according to Harrington et al. (2001).

Results

Basic water balance characteristics of nondiapausing females

Water balance characteristics determined in this experiment for C. pipiens exposed to no previous dehydration bouts were similar to those reported previously (Benoit and Denlinger, 2007; Benoit et al., 2009). The initial mass of the nondiapausing mosquitoes was 3.62 ± 0.13 mg, and they contained 2.41 ± 0.11 mg water (66.6% water content). Water loss rate was $3.26 \pm 0.09\%/h$, dehydration tolerance was $34.6 \pm 1.1\%$ and survival at 0% RH was 13.4 ± 0.8 h. No significant differences were noted in any water balance characteristics after mosquitoes were subjected to 4 bouts of dehydration (Fig. 2; ANOVA; P > 0.05). Following 6 bouts of dehydration, survival, dry mass and dehydration tolerance were significantly lower for water-only mosquitoes compared to sugar + water and fully hydrated mosquitoes (Fig. 2; ANCOVA; P < 0.05), and this trend continued after 8 and 10 bouts of dehydration (Fig. 2). By contrast, water loss rates and water mass were not significantly different at any point in experiments using nondiapausing C. pipiens (Fig. 2.; ANOVA; P > 0.05). Dehydration tolerance was the only factor that was significantly lower for the water-only group (Fig. 2; ANOVA, P <0.05). Based on these results, we conclude that survival of C. pipiens will be reduced following successive bouts of dehydration unless the mosquitoes have the opportunity to replenish their metabolic reserves.

Depletion of nutritional reserves by dehydration

The decline in dry mass, implies that metabolic reserves of C. pipiens were negatively impacted by multiple dehydration bouts. Initial lipid, carbohydrate, and glycogen levels in adult females were similar to those previously reported (Vaidyanathan et al., 2008; Sim and Denlinger, 2008). Reductions in glycogen and lipid were apparent after four bouts of dehydration for water-only mosquitoes (ANOVA; P < 0.05), whereas there was a significant increase in carbohydrate content for sugar + water mosquitoes after only two bouts of dehydration (Fig. 3). A significant reduction in protein content of the water-only mosquitoes was noted only after 8 bouts of dehydration (Fig. 3). By comparison to females provided with sugar between dehydration bouts, water-only control females exposed to 10 bouts of dehydration had reduced lipid levels by 62%, glycogen by 85%, protein by 24%, and sugar reserves were completely eliminated (Fig. 3). Lipids, glycogen and carbohydrates also significantly declined in fully-hydrated controls as time progressed (Fig. 3; ANOVA, P < 0.05), but these reduction were not as severe as observed in water-only mosquitoes subjected to bouts of dehydration. Thus, nutritional reserve declined when mosquitoes were not provided a sugar resource, and this effect was exacerbated by multiple dehydration exposures.

Impact of dehydration bouts on diapause

To determine if bouts of dehydration also impact diapausing mosquitoes in the same way, we monitored metabolic reserves and survival after exposures to different numbers of dehydration bouts. The first significant reduction in survival was noted after 10 dehydration bouts (Fig. 4a), and by that time lipid levels had dropped 36%, glycogen

content by 56%, and sugar content by 33%. After 25 dehydration bouts, lipid content was reduced by 55%, glycogen content by 84%, and sugar content by 96% (Fig 4a). Survival of diapausing *C. pipiens* was reduced by nearly 25% after 10 bouts and by nearly 50% when females were exposed to 25 bouts of dehydration (Fig. 4b). Thus, diapausing females of *C. pipiens* were affected in the same manner as observed in nondiapausing females: dehydration reduced metabolic reserves, resulting in reduced survival.

Egg production

Nondiapausing females laid significantly more eggs/raft than females that had been through diapause (234 ± 19 eggs vs. 175 ± 17 eggs). Five bouts of dehydration (nondiapause females) or 10 bouts (diapause females) reduced egg production compared to controls (Fig. 5). Nondiapausing mosquitoes that were offered sugar during their recovery period produced nearly the same number of eggs per raft as those that were not dehydrated (Fig 5, ANOVA, > 0.05). The mean carbohydrate (1.0 µg), glycogen (0.4 µg) and lipid (0.7 µg) content within each egg did not vary between treatment groups (ANOVA, P > 0.05). Thus, multiple bouts of dehydration did not influence reserves packaged within the egg, but it did have a significant effect on the number of eggs per raft, and this negative effect could be countered, at least in nondiapausing females, by provision of a sugar source.

Discussion

Nearly all previous studies on water balance in mosquitoes and other insects evaluated the impact of only a single exposure to dehydration (Hadley, 1994; Gray and Bradley, 2005; Benoit and Denlinger, 2007; Benoit et al., 2009). This is the first study to evaluate the impact of multiple bouts of dehydration and rehydration on mosquito physiology. In our experiments, each dehydration exposure was followed by subsequent rehydration. Several recent studies suggest that dehydration and rehydration elicit distinct stress responses (Hayward et al., 2004; Lopez-Martinez et al., 2009), thus rehydration cannot be viewed as a simple reversal of dehydration stress.

Our results demonstrate a progressive decline in glycogen, lipid and sugar content as the number of dehydration/rehydration bouts increased. This negative effect can be ameliorated by allowing the mosquito to ingest sugar between dehydration bouts. The reduction in nutritional reserves that we observed is likely the consequence of the mosquito's response to water stress. This form of stress invokes energy-depleting activities such as the mobilization of antioxidants, heat shock proteins (Lopez-Martinez et al., 2009), aquaporins, late embryogenesis abundant (LEA) proteins (França et al., 2005) and cytoskeletal proteins (Li et al., 2009). In a recent study, we showed that heat shock protein 70 (hsp70) and hsp90 are essential to the response of mosquitoes to dehydration stress (Benoit et al., 2009). In addition to changes in protein levels, other molecules including trehalose and glycerol are likely to be generated to prevent protein interactions and reduce membrane changes as water levels within the mosquito fluctuate (Goyal et al., 2005; Watanabe, 2004). Unless these reserves are replenished, multiple bouts of dehydration exhaust the glycogen, lipid, and sugar reserves, thus denying the mosquito the nutritional resources required to respond and subsequently recover from dehydration stress.

Nondiapausing females of *C. pipiens* have the potential to replenish their reserves by feeding on nectar and avian hosts that are readily available during the summer months. But, such is not the case for diapausing females. In response to short daylength of late summer and early fall, females of C. pipiens cease feeding on blood and rely exclusively on sugar sources to generate the reserves needed to bridge the winter months (Mitchell and Briegel, 1989; Bowen, 1992; Robich and Denlinger, 2005). The diapausing females retreat to protected habitats such as caves and culverts (Vinogradova, 2000) and thus spend the winter in locales that are unlikely to offer sugar resources. Thus, we suspect that diapausing females would be the stage most likely to experience bouts of dehydration, and also due to the absence of sugar resources during the winter, it is the stage likely to be most vulnerable to this form of stress. High humidity in the overwintering habitat is critical for survival, and our present set of experiments suggest that mosquitoes in areas prone to varying water availability would continually need to respond to changes in water content, thereby depleting energy reserves necessary to survive until diapause is terminated in the spring.

Bouts of dehydration impact not only survival of the adult, but also the number of eggs she produces. Females with reduced nutritional reserves are well known to produce fewer eggs (Foster, 1995; Ziegler and Ibrahim, 2001; Harrington et al., 2001; Zhou et al., 2004). Females allowed to feed on sugar prior to blood feeding have significantly higher lipid reserves, allowing these females to produce more eggs that contain more lipids

(Ziegler and Ibrahim, 2001; Zhou et al., 2004). During the first gonotrophic cycle, carbohydrate reserves, along with lipids, are a critical source of energy, thus a severe reduction in such reserves is likely to reduce egg production. Our experiments showed that carbohydrate, glycogen and lipid levels of adult females were negatively impacted by chronic bouts of dehydration bouts, resulting in a lower production of eggs in both nondiapausing females as well as in post-diapause females exposed to multiple dehydration bouts during diapause. Interestingly, no changes in the nutritional reserves were noted within individual eggs, but fewer eggs were produced, a result that is consistent with previous observations by Harrington et al. (2004) on *Aedes aegypti* where non-optimal bloodmeals reduced egg quantity rather that individual quality. Thus, our results indicate an indirect impact of water balance on egg production, presumably through the diversion of energy reserves from egg production to the combating of water stress.

In conclusion, we demonstrate that multiple bouts of dehydration and rehydration significantly impact the physiology of *C. pipiens*. We show that nutritional reserves are utilized in response to dehydration, resulting in the progressive erosion of dehydration resistance. This effect can be alleviated by sugar feeding between dehydration bouts. To the best of our knowledge, no previous studies have addressed the effect of numerous dehydration exposures on arthropods, but several studies have tested the impact of multiple freeze/thaw cycles in insects (Bale et al., 2001; Sinclair and Chown, 2005). Our results suggest that determining how an insect responds to chronic bouts of dehydration may be important in evaluating an insect's water balance profile. For insects undergoing dormancy, maintaining water balance is one of the most critical factors determining

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survival (Danks, 2000; Benoit, 2009). Most insects in diapause have reduced water loss rates (Benoit, 2009), but in this study we show that each bout of dehydration reduces the metabolic reserves needed to survive through the dormant period and this can impact post-diapause egg production. Thus, prevention of chronic dehydration during diapause may be critical for insects that do not feed during their dormant periods since these insects rely solely on nutritional reserves obtained before the onset of dormancy.

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Figure 4.1. Schematic representation of the experimental protocol, showing three bouts of dehydration and rehydration. ND, nondiapause; D, diapause; RH, relative humidity.


Figure 4.2. Basic water balance characteristics of nondiapausing adult females of *Culex pipiens* after multiple bouts of dehydration. \blacktriangle , water-only during recovery; \blacksquare , 10% sucrose and water during recovery; \blacklozenge , fully-hydrated controls. Each point is the mean \pm SE of 24 individuals. ^a, denotes significance (P < 0.05) between the three treatments, based on ANOVA.



Figure 4.3. Nutrient reserves in nondiapausing adult females after multiple bouts of dehydration. \blacktriangle , water-only during recovery; \blacksquare , 10% sucrose and water during recovery; \blacklozenge , fully-hydrated controls. Each point is the mean ± SE of 24 individuals. ^a, denotes significance (P < 0.05) between the three treatments groups based on ANOVA.



Figure 4.4. Changes in (A) nutrient reserves and (B) survival of diapausing adult females after multiple bouts of dehydration. \blacktriangle , sugar; \blacksquare , lipids; \blacklozenge , glycogen. Each point is the mean \pm SE of 15 individuals. Superscripts denote significance (P < 0.05).



Figure 4.5. gg production in (A) nondiapausing and (B) diapausing adult females following blood ingestion after multiple bouts (N = 0-20) of dehydration. Each point is the mean ± SE of 15 individuals. Superscripts denotes significance (P < 0.05).

Chapter 5: Water balance of the bed bug, Cimex lectularius

Overview

This chapter consists of two separate studies on water balance aspects of the bed bug, *Cimex lectularius*. The recent emergence of bed bugs has prompted a great deal of research on this pest, particularly how this insect survives extended periods between bloodmeals. The first study encompasses the basic water requirements of all stages, except the eggs, of *C. lectularius*. Based on these results, I conclude that bed bugs are adapted for xeric conditions due to their low water loss rates andtheir use of clustering to reduce water loss. The second study investigates utilizing desiccant dusts to control bed bugs. Particularly, we examined if the addition of bed bug alarm pheromone components, (E)-2-hexenal and (E)-2-octenal, increases the effectiveness of desiccants. Addition of the alarm pheromone components increased mortality induced by desiccant dust by substantially increasing water loss rates. Overall, these two studies provide the first complete description of bed bug water balance and a mechanism for utilizing desiccant dusts plus alarm pheromones to increase water loss and decrease survival of *C. lectularius*. Resistance to dehydration between bouts of blood feeding in the bed bug, *Cimex lectularius*, is enhanced by water conservation, aggregation, and quiescence.

Abstract

To determine how the bed bug, Cimex lectularius, survives in a dry environment for many months without feeding, water-balance characteristics were compared for all stages from first-instar nymphs to adults. This species is characterized by a low net transpiration rate averaging < 0.2%/h, high tolerance for dehydration (30–40% loss in body water), and an impermeable cuticle as indicated by a high critical transition temperature (CTT) in the 35–40°C range, implying that this insect is adapted for desiccation-hardiness. The capacity of adults to survive for 2 weeks at 0.00*a*v ($a_v = \%$ RH/100) with no access to food or water exemplifies this trait. In contrast to more mature stages, first-instar nymphs contain more water, lose water at a faster rate, experience abrupt water loss at a lower temperature, and survive less time in dry air, suggesting that this stage is the most sensitive to water stress. This insect relies on blood to replenish water stores; none of the stages examined have the capacity to absorb water vapor (critical equilibrium activity, $CEA > 0.99a_v$), and they drank only sparingly when offered free water. As the bed bugs progress through their development, they gradually reduce their water requirements while increasing their desiccation resistance. Surviving water stress is considerably enhanced behaviorally by quiescence, characterized by prolonged periods of inactivity, and by the formation of clusters that generate a water-conserving group effect.

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Introduction

The common bed bug, *Cimex lectularius*, has a remarkable ability to survive 4 months to 2 years without feeding (Usinger, 1966), a feature that presumably accounts for their incredible capacity to persist for long periods in human bedding and other locations. Recently, there has been a resurgence of bed bugs in human dwellings, including hotels (Reinhardt and Siva-Jothy, 2007; Doggett et al., 2004; Hwang et al., 2005). The prolonged absence of a suitable host is problematic for these obligate blood feeders due to a lack of fluid uptake to counter desiccation (Johnson, 1940; 1941). How do bed bugs cope with this challenge? Besides cannibalism (Usinger, 1966), no other attributes have been described that would account for their impressive survival capacity between bouts of blood feeding. Despite the current bed bug epidemic, few studies, with the exception of earlier works by Johnson (1940; 1941) and those summarized in Usinger (1966), have focused on the physiology of bed bugs, especially their mechanisms for enduring prolonged periods of fasting.

Epidemiologically, *C. lectularius* is a nuisance pest to humans, causing loss of sleep due to annoying bites (Reinhardt and Siva-Jothy, 2007; Doggett et al., 2004; Hwang et al., 2005). The bed bug does not appear to be a disease vector, but it has been associated with iron deficiency, secondary bacterial infection from bite sores, and allergic hypersensitivity (Feingold et al., 1968; Jupp et al., 1991; Blow et al., 2001). This species is broadly distributed, occurring throughout much of the

temperate zone, and is most successful in cosmopolitan areas with high human density (Usinger, 1966). All five nymphal stages and adults are mobile and require a blood meal to molt, and the adult requires an additional blood meal to reproduce (Reinhardt and Siva-Jothy, 2007). Bed bugs remain hidden during the day, and because of their small size, lack of wings, and flat body shape, they are able to crawl into tight crevices. After feeding forays, the bugs return to these sites, resulting in the formation of dense aggregations (mixed stages), known as "brood centers," where eggs, fecal material, and exuviae also accumulate (Usinger, 1966; Reinhardt and Siva-Jothy, 2007). When in these clusters, the bugs enter a quiescent state while the blood meal is digested; they venture out again only for host seeking when their metabolic reserves have been depleted (Usinger, 1966; Mellanby, 1932; Mellanby, 1939).

Previous research, discussed in a review by Johnson (1941), on the water requirements of *C. lectularius* indicates that this bug is particularly tolerant of drying (Usinger, 1966; Johnson, 1940; 1941), but factors influencing their unique dehydration resistance and how these factors may contribute to the recent proliferation of bed bugs have not been determined. In this study, we construct a water balance profile for *C. lectularius* with the goal of examining habitat preference and suitability, features that are critical for survival and for determining the bug's potential to spread into new regions. We examine the entire life cycle (except eggs), using bugs of similar age to illustrate developmental shifts in water requirements and to pinpoint the stages that are most and least vulnerable to water stress. We assess percentage body water content, dehydration tolerance limit, net transpiration rate (integumental plus respiratory water loss), critical transition temperature (CTT, denoting the temperature threshold of an abrupt lethal water loss), and free water drinking ability. The benefit of clustering for water conservation was evaluated by measuring net transpiration rates of individual bugs and of different-sized groups. Rivnay (1932) and Johnson (1941) showed prolonged survival of various bed bug stages at conditions of water deficiency that are similar to those regularly encountered in human dwellings (30–50% RH and 22–24°C based on comfort standards; Reinikainen et al., 1992; Ferng and Lee, 2002) thus suggesting that these bugs may have the capacity to absorb water vapor from the air. An additional goal of our study was to determine the bed bug's critical equilibrium activity (CEA), to test if the bugs can use atmospheric moisture as a primary source of water.

Materials and Methods

Bed bugs and test conditions

C. lectularius was acquired from The Ohio State University Insectary. The colony was established in 2002 from individuals collected in Columbus, OH. Bugs were stored at 85% RH, 15 h:9 h light/dark until they were used for these experiments. Temperature was $25 \pm 1^{\circ}$ C for the colony and also for basic observations; this temperature allows us to compare our results with previous studies on insect water balance (Hadley, 1994). Each of the five nymphal stages and male and female adults were used in the experiments. All individuals were used 1 week after molting or after hatching in the case of the first instar. An aspirator and felt-tipped soft forceps were

used for transferring the bugs and for handling the bed bugs during mass measurements.

Test relative humidities (% RH) were generated with the use of saturated salt solutions (33% RH with MgCl₂, 75% with NaCl, 85% with KCl, 93% with KNO₃, 98% with K₂SO₄) as described by Winston and Bates (1960), distilled water (100% RH) or calcium sulfate (CaSO₄, 0% RH; 1.5×10^{-2} % RH) that was placed in the base of sealed glass desiccators (5,000 cc). A hygrometer (SD ± 0.5% RH; Thomas Scientific, Philadelphia, PA) was used to verify each experimental relative humidity. To relate the water present within the bugs to that in the surrounding atmosphere, relative humidities were expressed as water vapor activities (a_v ; $a_v = \%$ RH/100; thus, $0.00a_v$, $0.33a_v$, $0.85a_v$, $0.93a_v$, $0.98a_v$ and $1.00a_v$) and the activity of the body water (a_w) of the bug > $0.99a_w$ based upon mole fraction (Toolson, 1978). Bed bugs were housed individually within 1-cc mesh-covered chambers that were placed on perforated porcelain plates to prevent contact with the solution used to generate the water-vapor activities.

Bugs were weighed individually using an electrobalance (CAHN; SD \pm 0.2 µg precision and \pm 6 µg accuracy at 1 mg; Ventron Co., Cerritos, CA) without enclosures and without the use of anesthesia. Briefly, a bug was removed from its enclosure and permitted to crawl onto the weighing pan of the balance, the mass was determined, and the bug was picked up with an aspirator and returned to the 1-cc mesh enclosure and test conditions. This was accomplished in < 1 minute. Before being used in experiments, the bugs were held at $0.33a_v$ until a 4–6% loss in body weight occurred, thus minimizing the effect of excretion, digestion, and reproduction on mass changes

(Wharton, 1985; Arlian and Ekstrand, 1975) and standardizing the bugs with regard to water flux so that mass changes only reflected internal fluctuations of the bug's water content. At the end of each experiment, bugs were placed at 90 °C and $0.00a_v$ and monitored until mass became constant, then held for an additional 3 days of drying; this mass was then recorded as the dry mass.

Water balance characteristics.

Wharton's methods (1985) and equations, with modifications by Yoder and Spielman (1991), Kahl and Alidousti (1997), and Benoit and others (2005), were used to determine the various water balance characteristics. Dry mass (d) was subtracted from initial (fresh) mass (f) to determine the amount of water that is available for exchange, which is defined as the water mass (m). Water mass was expressed as a percentage of the initial mass to determine the percentage body water content. Bugs were placed at $0.33a_v$ and 30° C, weighed every hour, and tested for their ability to right themselves and crawl five body lengths. The mass measurement that corresponded to the point where they were unable to achieve this behavioral task was defined (after subtracting corresponding dry mass) as the critical mass, mc, and was used as an estimate of dehydration tolerance based on the percentage change in mass lost from initial to critical mass (m_c).

To determine net transpiration rate (= integumental plus respiratory water loss), bugs were weighed, placed at $0.00a_v$ and reweighed at various intervals, for a total of five readings of mass; weighing intervals varied between instars depending on the extent of their desiccation. Net transpiration rate was determined at $0.00a_v$ because the amount of water mass lost declines exponentially such that water loss rate can be derived from the slope of a line described by the equation: $m_t = m_0 \exp(-kt)$, where m_t , is the water mass at any time t, m_0 is the initial water mass, and -kt is the rate of transpiration expressed as %/h. Rates were established for isolated individuals and also for individuals in groups of different sizes. Individuals were marked on the dorsum with a spot of paint (Pactra, Van Nuys, CA) and allowed to cluster naturally. The paint-marked bugs were removed for mass determinations and then returned to the group; paint had no effect on mass changes (data not shown). Critical transition temperature (CTT), the temperature threshold of a rapid water loss, was based on change in activation energy (E_a) determined by analyzing water-loss rates over a broad temperature range (4–60°C) as described by the Arrhenius equation: k = A $\exp[-E_a/(RT)]$, where k is the net transpiration rate, A is steric (frequency) factor, T is absolute temperature, R is gas constant, with E_a based on the slope, which is equal to $-E_a/R$.

Avenues of water gain were also investigated. Drinking free water was analyzed by offering bugs 5- to 20- μ L droplets of 0.5% Evans blue-stained water in a 100 x 15 mm petri dish (0.75% RH, 25°C). Ten bugs were placed in each dish, examined for 20 minutes (40× microscopy), and then every 2 hours thereafter. After 24 hours of observation, bugs were dissected in 0.1% NaCl under the microscope (100x) and examined for the presence of blue coloration in their digestive tract and liberation of dye when the gut was opened. Water vapor absorption was examined by long-term daily monitoring of water mass (*m*) of individual bugs held at different water-vapor activities. The capacity to maintain a steady water mass in subsaturated air (< 0.99aw of the bug's body water), thus balancing water loss with water gain from the air (water gain > water loss), was taken as evidence of the bug's ability to use atmospheric water vapor as a primary source of water. Survivorship was assessed based on 40x microscopic observations of dead bugs; bugs were considered dead if they were immobile, unable to right and crawl, and failed to respond to prodding or bright light.

Sample sizes and statistics

Each experiment was replicated 3 times with 10 bed bugs per replicate, for a total of 30 individuals for each water-balance characteristic determination. Data, reported as the mean \pm SE, were compared with analysis of variance (ANOVA), and arcsin transformation was used in the case of percentages (Sokal and Rohlf, 1995). A test for the equality of slopes of several regressions was used to compare characteristics derived from regression lines. Survivorship times were compared using *t* statistics.

Results

Water content

Initial mass, dry mass, and water mass increased with each successive stage (Table 5.1). Percentage body water content was highest for first-instar nymphs (71%) and lowest (67%) for female adults (ANOVA; P < 0.05). No significant difference in percentage body water content was observed between sexes (ANOVA; P > 0.05). In all cases, within a particular stage, the water mass was a positive correlate of dry

mass, with R = 0.93, 0.89, 0.94, 0.93, 0.95 for first- through fifth-instar nymphs, respectively, 0.96 for adult males and 0.95 for adult females (ANOVA; P < 0.001). Corresponding water mass to dry mass values through the series of first- through fifth-instar nymphs were 2.35, 2.31, 2.28, 2.24, and 2.20, respectively, showing a progressive decline as dry mass increased in proportion to water mass. Water mass to dry mass ratios for adults were 2.13 for males and 2.04 for females. Within a particular stage, individuals were similar in size and shape and water content, indicating that surface area to volume properties are standardized; thus each stage can be anticipated to exhibit a consistent, stage-related water flux. We conclude that the proportion of body water is highest for bed bugs in their earliest stages of development.

Net transpiration rate.

First-instar nymphs lost water (net transpiration rate) at a rate of $0.402 \pm 0.011\%$ /h (Table 5.1). The corresponding rate of water loss for individuals (paint marked) in a group of 20 (0.247 ± 0.010%/h) was approximately half that of isolated individuals (Table 5.1; Figure 5.1; ANOVA; P < 0.05). Net transpiration rates of first-instar nymphs in groups of 5 (0.360 ± 0.014%/h) and 10 (0.311 ± 0.017%/h) were between these extremes (ANOVA; P < 0.05). In all cases, the paint-marked individuals were returned to the group after mass determination, and they remained in the group. It is also important to note that members of the groups did not disband when disturbed by removing or reintroducing the paint-marked individuals and individuals were in direct contact with neighboring bugs. Periodically, the location of

the bed bugs was recorded and movement was noted; these observations suggested that individuals likely spend nearly equal time in the middle and at the edges of the group. Net transpiration rates of other stages are presented in Table 5.1 and follow a similar exponential pattern of water loss (R > 0.99; ANOVA; P < 0.001), reflecting proportionate loss at $0.00a_v$ on a semilogarithmic plot as illustrated by first-instar nymphs in Figure 1. Net transpiration rate increased with each successive stage during development (Table 1; ANOVA; P < 0.05). Net transpiration rate also correlated with dry mass (y = -0.38x, R = 0.94; ANOVA; P < 0.001; Figure 5.2), indicating that water loss varies according to body size. Net transpiration rate was 4× higher for first-instar nymphs than adult females, a stage that is nearly 50x larger (Table 5.1). We conclude that water is lost most rapidly when the surface area is greatest relative to volume and that a strong, positive relationship exists between aggregation size and suppression of water loss.

Critical transition temperature

Net transpiration rate of first-instar nymphs increased with increasing temperature and exhibited a Boltzmann temperature function (R > 0.95; ANOVA; P < 0.001; Figure 5.3). A distinct critical transition temperature (CTT) was detected in these first-instar nymphs as evidenced by a steep slope of the regression line indicative of a new temperature range (biphasic, two-component curve) and a change in activation energy (E_a). The change in slope was due to higher proportionate amounts of water loss in the higher temperature range. The CTT of first-instar nymphs was 34.5°C, as determined by identifying the point of intersection of the two regression lines plotting the E_a changes. Other stages responded similarly to temperature and yielded nearly identical net transpiration rate-temperature relationships as shown for first-instar nymphs in Figure 3; the results indicate Boltzmann dependence on temperature (R > 0.95; ANOVA; P < 0.001), different proportionate water mass losses in low and high temperature ranges leading to a change in slope, and evidence for a CTT. The CTT increased through development from 34.5°C for first-instar nymphs to 39°C for female adults (Table 5.1; ANOVA; P< 0.05). Compared with adults, earlier stages are more at risk of abrupt, rapid desiccation at high temperature.

Dehydration tolerance

When examined under the microscope (40x), bed bugs remained immobile and failed to respond to prodding for 15–20 seconds, then slowly uncurled their legs, righted, and began to crawl. Once having lost about 1/3 of their water content, they failed to right themselves and crawl 10 body lengths, as indicated by critical mass (m_c) values (Table 5.1). No significant differences were observed among the various stages when critical mass was expressed as a percentage of the amount of body water that was lost, averaging 35% (Table 5.1; ANOVA; P > 0.05). Throughout their life cycle, bed bugs tolerated similar levels of dehydration stress.

Survivorship

Female adults were capable of surviving a remarkable 16 days (50% of adults) at $0.00a_v$ with no food or water, demonstrating their ability to withstand prolonged periods of starvation and desiccation. Females survived approximately 2 days longer than males (t statistics; P < 0.05). These survivorship estimates for adults agree well with our calculated dehydration tolerance limits and net transpiration rates (Table 5.1). Similarly, the time required to reach critical mass (dehydration tolerance limit) calculated from net transpiration rates for each of the immatures closely matched length of survival in dry air (Table 5.1; t statistics; P < 0.05). Thus, the relationship between net transpiration rate, dehydration tolerance, and length of survival in dry air are consistent for all stages throughout the life cycle. Once they reached their critical mass, the bugs were unable to be rescued by placing them at $1.00a_v$ or by offering them droplets of free water (each N = 10/stage), thus indicating that they had sustained an irreversible level of dehydration. None of the bugs in this condition survived. Our results show that adults are more resistant to desiccation than immatures.

Free water drinking

When bed bugs were placed into a petri dish containing droplets of Evans blue-stained water, they crawled about quite actively as though engaged in a searching type of behavior, characterized by tapping the bottom of the dish with the antennae as they moved about the arena. Within 1–2 minutes, most of the movement stopped and the bed bugs were observed to be clustered around the edges of the dish.

Little movement was observed within these aggregations or to adjacent clusters. In all cases, water droplets were encountered passively, and none of the bugs displayed deliberate movements away or toward the droplets of water. They did not appear to be attracted or repelled by the droplets. On occasion, upon encountering a droplet of water, the bug paused, inserted its proboscis into the droplet and drank; this lasted for approximately 1 minute (Table 5.1). This set of behavioral observations included each of the various stages examined. Blue dye could be seen filling the gut diverticula when the bug was observed under the microscope (40x), and blue coloration was liberated from the gut upon dissection (100x), a confirmation that the bugs consumed the colored liquid. Attempts to encourage the bugs to drink by dehydrating them by losses of 20–25% of their body mass (0.00 a_v and 30°C) prior to exposing them to the water droplets were futile. Instead, these dehydrated bed bugs formed clusters and only a few were observed to imbibe the water and the water droplets were still encountered passively; that is, no deliberate movements were made to the droplets. Although bed bugs have the ability to drink water, they appear to do so rather sparingly.

Water vapor absorption

First-instar nymphs failed to maintain their water mass over a period of several days when exposed to subsaturated air (Fig. 5.4). This was due to the activity gradient created between the activity of the body water ($0.99a_w$; Wharton, 1985) with that of surrounding air, thus $0.99a_w > 0.98a_v$, $0.93a_v$, $0.85a_v$, and $0.75a_v$, and this resulted in water loss by simple diffusion. Less net water loss was measured as the air

became more humid (R > 0.97; ANOVA; P < 0.001), which demonstrated an effect of greater passive adsorption onto the surface of the cuticle as water vapor activity approached saturation. Water gain occurred and the uptake was maintained in saturated air of $1.00a_v$ because the activity gradient was reversed, $1.00a_v > 0.99a_w$ (body water), and this drove water inward into the bug's body and contributed to water mass. Because net water loss occurred at $0.98a_v$ and net water gain at $1.00a_v$, water balance was achieved (gain =loss) at $0.99a_v$, which is equivalent to the $0.99a_w$ body water activity estimated by Wharton (1985). Accordingly, the critical equilibrium activity (CEA) of first-instar nymphs was $> 0.99a_v$. Other stages displayed a similar pattern of water loss in subsaturated air and gain at saturation consistent with properties of diffusion as illustrated by Figure 5.4 for the first-instar nymph. For all stages, CEA $> 0.99a_v$ (Table 5.1). Thus, bed bugs cannot use water vapor as a significant source of water.

Discussion

Outstanding water balance features of *C. lectularius* include a low net transpiration rate (< 0.2%/h) that is similar to rates observed in desert-adapted arthropods, an ability to tolerate loss of 1/3 of their body water (most arthropods only tolerate 20–30% loss), and a high (> 35°C) critical transition temperature consistent with arthropods that have water impermeable cuticles (Hadley, 1994; Wharton, 1985; Edney, 1977). This set of water-balance characteristics is typical for arthropods that are differentially adapted for life in a dry habitat according to Hadley (1994). Thus, *C*. *lectularius* is xerophilic with regard to water balance. As such, *C. lectularius* is modified for water conservation and to resist desiccation, wherein their ability to retain water (low water loss rate) is more important than their ability to gain water. Other water-balance characteristics of *C. lectularius*, such as the \approx 69% body water content, free water drinking, and lack of ability for water vapor absorption are similar to features observed in most arthropods (Hadley, 1994). In fact, as adults, the characteristics of *C. lectularius* resemble those of another bloodfeeding hemipteran, *Rhodnius prolixus* (69% water content, 50% dehydration tolerance, low water loss, xeric ecologic classification; Hadley, 1994; Gringorten and Friend, 1982; Gringorten and Friend, 1989). Notably, emphasis on water retention and desiccation-resistance properties by *C. lectularius* enables this species to function effectively in a dry environment and dually protects the bugs against desiccation between bloodmeals or in the absence of a host.

The fact that *C. lectularius* failed to absorb water vapor is not unusual, as most arthropods lack this ability (Hadley, 1994; Winston and Bates, 1960). Arthropods that achieve this feat maintain body water levels at otherwise dehydrating conditions by absorbing water against the atmospheric gradient using a mechanism frequently involving a salt-driven process (O'Donnell and Machin, 1988; Gaede and Knülle, 1997). Because water vapor absorption does not occur in *C. lectularius*, the long-term survivorship in the absence of food and in relatively dry air, reported by Rivnay (1932) and Johnson (1941) must be the consequence of water conservation properties rather than the ability to use water vapor from the air. For all stages, the CEA > $0.99a_v$, indicating that water gain can only occur from air that is completely water-saturated at $1.00a_v$, a water vapor activity that corresponds to water as a liquid. This necessarily implies that water must be imbibed in fluid form (Arlian and Eckstrand, 1975). In the presence of free water, however, stages of *C. lectularius* were not attracted to droplets of water and only drank on occasion, displaying no real interest in the water even when they were dehydrated. Such inconsistent drinking patterns were similarly observed in certain ticks (Kahl and Alidousti, 1997) and are typical of arthropods that feed on blood (Hadley, 1994). Indeed, blood feeding is responsible for the majority of the contributions to the internal body water content of *C. lectularius* (Mellanby, 1932), as well as *R. prolixus* (Heger et al., 2006), a species that shares many water-balance characteristics with *C. lectularius*. Although free water represents a viable water resource, the most likely primary source of water for *C. lectularius* is their blood diet.

Other noteworthy features related to water balance in *C. lectularius* are primarily behavioral regulators of water loss due to the capacity to enter long-term quiescence and the ability to form clusters. The quiescence is characterized by a complete lack of activity, including retraction of the legs that give the bugs a dead appearance. This quiescence can only be broken by persistent prodding and provoking. Clearly, this shut-down makes a major contribution to conservation of the body water content by helping to reduce respiratory water loss (Hadley, 1994), an ability that is similar to that seen in the spider beetle, *Mezum affine*, a species that can survive without water for many months (Benoit et al., 2005). Suppression of net transpiration rate is common in arthropods that aggregate, including mites (Glass et al., 1998), beetles (Yoder and Smith, 1997), and cockroaches (Yoder and Grojean,

1997). A well-known additional benefit of aggregation is to increase access to mates (Yoder and Smith, 1997), and this is consistent with the designation of the bed bug cluster as a "brood center." Although our experiments on the group effect were restricted to first-instar nymphs, it is likely that all stages experience and benefit from this effect. Our preliminary results using flowing dry air suggest that the mechanism of the group effect operates by generating a humidified boundary layer, as noted in beetles (Hodek, 1973). Linkage between quiescence and enhanced water conservation is similar to that observed during overwintering diapause (Denlinger, 2002). This feature raises the yet untested possibility that quiescent stages of *C. lectularius* may also be cold tolerant.

Developmentally, the stage of *C. lectularius* that is most sensitive to desiccation stress is the first-instar nymph. This stage has the highest percentage body water content, indicating that it requires more body water to function. The first instar nymph is also the smallest in body size, thus making it more vulnerable to rapid water loss rate due to its surface area to volume properties. Greater cuticular permeability to water is implied by a lower CTT, suggesting that the cuticular structure of the first instar is more easily disrupted. The high water requirement must be met by increased feeding activity or a greater reliance on clustering. Stage differences in water requirements are unlikely to be indicators of a different habitat preference because all instars are found clumped together within the same microhabitat. Through development, percentage body water content declines concurrently with an increase in dry mass (= fat); thus, the bugs require less water and accumulate greater fat reserves as they become older. In addition, as the bed bugs proceed through

development, they increase in body mass (size), allowing for greater water retention, and the CTT increases by 5°C, implying that the integument becomes progressively more water-tight. Thus, there is a gradual shift through development from a high to a low water requirement, and this is reflected by differences in survival enabling adults to live the longest in a desiccating environment.

From this study it is clear that the water balance strategy of *C. lectularius* emphasizes water retention. These effective water-conserving traits are supplemented behaviorally by a quiescence marked by periods of inactivity and a group effect that enhances protection against desiccation stress. Effective water conservation thus enables the bed bugs to persist for long periods without rehydration. It would appear that the human comfort standards of $0.30-0.50a_v$ and $22-24^\circ$ C (Ferng and Lee, 2002) create an ideal habitat for *C. lectularius*. As long as a host is available on occasion for blood feeding, the species is well adapted to survive the water balance challenges encountered in most human dwellings.

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Characteristics	Nymphs					Adults	
	1st	2nd	3rd	4rh	5th	Male	Female
Water content							
Initial mass, $f(mg)$	0.124 ± 0.023	0.255 ± 0.016	0.821 ± 0.037	1.323 ± 0.041	2.621 ± 0.044	4.892 ± 0.037	5.472 ± 0.046
Dry mass, d (mg)	0.37 ± 0.009	0.077 ± 0.011	0.250 ± 0.021	0.408 ± 0.031	0.819 ± 0.032	1.561 ± 0.029	1.805 ± 0.032
Water mass, m (mg)	0.087 ± 0.010	0.178 ± 0.021	0.571 ± 0.033	0.912 ± 0.036	1.802 ± 0.034	3.331 ± 0.041	3.677 ± 0.052
Water content (%)	70.9 ± 2.1	69.8 ± 1.7	69.5 ± 1.4	69.1 ± 1.9	68.8 ± 2.2	68.1 ± 1.8	67.2 ± 2.6
Water loss							
NTR (%/h; Figure 1)	0.402 ± 0.011	0.322 ± 0.019	0.181 ± 0.007	0.152 ± 0.018	0.121 ± 0.009	0.101 ± 0.007	0.091 ± 0.011
m_c (mg)	0.054 ± 0.004	0.112 ± 0.011	0.364 ± 0.016	0.589 ± 0.026	1.196 ± 0.031	2.241 ± 0.021	2.394 ± 0.036
DT (%)	37.4 ± 4.6	36.8 ± 4.1	36.2 ± 2.8	35.4 ± 2.9	33.6 ± 2.6	32.9 ± 0.9	34.9 ± 1.5
Survivorship at 0.00a.	3.9 ± 0.9	4.8 ± 0.6	8.3 ± 1.1	9.8 ± 1.2	11.6 ± 0.6	13.6 ± 0.8	16.0 ± 1.5
CTT (°C; Figure 3)	34.5 ± 2.4	35.9 ± 2.2	36.7 ± 1.9	37.6 ± 2.2	38.2 ± 1.6	38.9 ± 2.0	39.2 ± 1.1
Water gain							
Free water uptake	+	+	+	+	+	+	+
CEA (Figure 4)	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_v$	$\geq 0.99a_{\rm v}$	$\geq 0.99a_v$	$\geq 0.99a_{\rm v}$

* f, fresh (initial mass); d, dry mass; m, water mass; %, percentage body water content; m_c , critical water mass where they were unable to right and crawl; NTR, net transpiration rate; DT, dehydration tolerance as percentage lost to m_c ; CTT, critical transition temperature; survivorship is based on 50% of the bugs; drinking confirmed by presence of blue tracer in water droplet upon dissection; and CEA (critical equilibrium activity), the range of water vapor activities ($a_v - \%$ RH/100) where water vapor absorption occurs. Data shown are means \pm SE, n - 30.

Table 5.1. Comparison of water balance characteristics for different stages of *Cimex lectularius**



Figure 5.1. Proportion of water mass lost at $0.00a_v$ ($a_v = \%$ RH/100) and 25°C in firstinstar nymphs of *C. lectularius* as single isolated individuals and as an individual in a group of 20 first instars. The slope of the regression line is the rate of water loss (= net transpiration rate, integumental plus respiratory water loss). m_t , water mass at any time *t*; m_0 , initial water mass.



Figure 5.2. Relationship between net transpiration rate (water loss rate) and body size (dry mass) in stages of *C. lectularius*. Numbers above symbols on graph correspond to the various nymphal stages; M, male; F, female. Note high degree of fit of the regression line to a straight line ($R \approx 1.00$).



Figure 5.3. Water retention (net transpiration rate) as a function of temperature in first-instar nymphs of *C. lectularius*. Critical transition temperature (CTT), where water loss increases abruptly, is detected indicated by the point of intersection denoting change in slope of the regression lines (two-component curve) corresponding to a change in the activation energy (E_a) for water loss.



Figure 5.4. Changes in water mass of first-instar nymphs of *C. lectularius* in response to different water vapor activities (a_v ; $a_v = \%$ RH/100) and 25°C. Inability to maintain a steady water mass in dynamic equilibrium (water gain \neq water loss) in subsaturated air indicates that they do not actively absorb water vapor from the air.

The Addition of Alarm Pheromone Components Improves the Effectiveness of Desiccant Dusts Against the Bed Bug, *Cimex lectularius*

Abstract

We demonstrate the addition of bed bug, *Cimex lectularius*, alarm pheromone to desiccant formulations greatly enhances their effectiveness during short-term exposure. Two desiccant formulations, diatomaceous earth (DE) and Dri-die (silica gel), were applied at the label rate with and without bed bug alarm pheromone components, (E)-2-hexenal, (E)-2-octenal, and a (E)-2-hexenal:(E)-2-octenal blend. First instar nymphs and adult females were subjected to 10 minute exposures, and water loss rates were used to evaluate the response. Optimal effectiveness was achieved with a pheromone concentration of 0.01M. With Dri-die alone, the water loss was 21% higher than in untreated controls, and water loss increased nearly 2xwith (E)-2-hexenal and (E)-2-octenal, and 3x with the (E)-2-hexenal: (E)-2-octenal blend. This shortened survival of first instar nymphs from 4d to 1d, with a similar reduction noted in adult females. DE was effective only if supplemented with pheromone, resulting in a 50% increase in water loss over controls with the (E)-2hexenal: (E)-2-octenal blend, and a survival decrease from 4d to 2d in first instar nymphs. Consistently, the addition of the pheromone blend to desiccant dust was more effective than adding either component by itself, or by using Dri-die or DE alone. Based on observations in a small microhabitat, the addition of alarm

pheromone components prompted bed bugs to leave their protective harborages and to move through the desiccant, improving the use of desiccants for control. We concluded that short exposure to Dri-die is a more effective treatment against bed bugs than DE, and that the effectiveness of the desiccants can be further enhanced by incorporation of alarm pheromone. Presumably, the addition of alarm pheromone elevates excited crawling activity, thereby promoting cuticular changes that increase water loss.

Introduction

Alarm pheromones typically prompt a frenzied, rapid dispersal reaction of nearby conspecifics (Blum 1985). In the bed bug, *Cimex lectularius*, the active ingredients of the alarm pheromone are (E)-2-hexenal and (E)-2-octenal, present as a 75:25 blend in adults and a 30:70 blend for first instar nymphs (Levinson et al., 1971;1974). At low concentrations, (E)-2-hexenal and (E)-2-octenal, along with other chemicals, modify bed bug behavior by promoting an aggregation response (Siljander et al., 2008), which is responsible for retaining bugs of mixed stages in protected cracks and crevices (Usinger, 1966), facilitating water balance (Benoit et al., 2007), and enhancing reproduction by serving as 'brood centers' (Reinhardt and Siva-Jothy, 2007). An alarm response elicited by mechanical disturbance or agitation also prompts the release of (E)-2-hexenal and (E)-2-octenal, but in this case higher concentrations are released. In addition to a role of alarm pheromone in defense and alarm (Usinger, 1966), our recent results suggest that these same chemicals break quiescence and prompt the bed bugs to depart from their aggregations. The bed bugs are subsequently guided to their hosts by body temperature and other, albeit undefined, kairomones (Usinger, 1966). The available evidence thus indicates that (E)-2-hexenal and (E)-2-octenal in high concentration function as a general excitant, but at low levels function as part of the aggregation pheromone.

Historically, one of the most widespread tools for insect control are desiccant dusts, diatomaceous earths and silica gels (Ebeling, 1971). Although controversial (Korunic, 1998; Subramanyam and Roesli, 2000), most workers agree that these dusts cause damage to water proofing cuticular lipids, resulting in death by rapid desiccation (Allan and Patrican, 1994; Appel et al., 1999). Diatomaceous earth (DE) is usually regarded as an abrasive that scratches the cuticular surface and Dri-die (silica aerogel) is a non-abrasive and sorptive agent that adsorbs the cuticular lipids from the cuticular surface. The net effect of both agents is the same: cuticular lipids are removed and the water-proofing capacity of the cuticle is diminished (Appel et al., 1999). Desiccant dusts tend to lose their effectiveness at high relative humidities (> 81%) or in the presence of free water; dry human comfort standards of 30-50% RH and 22-24°C (Reinikainen et al., 1992; Ferng and Lee, 2002) are thus compatible with the use of desiccant dusts for bed bug control. Elevating temperatures is one effective strategy to increase insect movement and thereby increase contact with a desiccant (Athanassiou et al., 2005). In this study, we show that another tool for increasing contact with diatomaceous earth and Dri-die (abrasive vs. sorptive, respectively) is to augment these agents with the addition of bed bug alarm pheromones.

Materials and Methods

Bed bug culture

Cimex lectularius originated from specimens collected in Columbus, Ohio (Dublin Strain) and also from the National Pest Control Association (NPA strain). Cultures were maintained at 85% RH (using saturated salt solutions; Winston and Bates 1960), 15h:9h L:D, $25^{\circ}C$ ($\pm 1^{\circ}C$) and were reared on chicken blood by membrane feeding (Montes et al. 2002). All experiments were performed on first instar nymphs, one week after hatching, and on females one week after adult eclosion. All observations and experiments were conducted in the dark in red light. Bugs were handled with aspirators and felt-tipped forceps.

Petri plate tests for short-term exposure

Exposure methods and experimental design were based on procedures described by Allan and Patrican (1994), Arlian and Vyszenski-Moher (1995) and Appel et al. (1999). (E)-2-hexenal and (E)-2-octenal were from Sigma (> 99.9% purity; Sigma Chemical Co., St. Louis, MO) and were diluted in HPLC-grade acetone to 0.1M, 0.01M and 0.001M. A blend of (E)-2-hexenal:(E)-2-octenal (75:25 for adult and 30:70 for first instar nymphs based on glandular contents; Schildknecht, 1964; Levinson and Bar Ilan, 1970) was also tested at those same concentrations. Acetone served as a control. All solution preparations and applications were done in glass. Filter paper used in the experiments was No. 3 Whatman (Whatman, Hillsboro, OR). Diatomaceous earth (6.1g/m² label rate; 90% silica; Celite 545; Fisher Scientific, Pittsburgh, PA) and Dri-die (6.1g/m² label rate; 95% amorphous silica aerogel, 2% ammonium fluosilicate; Roussel Environmental Health, Frenchtown, NJ) were used as the desiccant dust formulations and were used at the label rate. Treatment exposures were conducted in 100 x 15mm sterile disposable Petri dishes with perforated lids (Fisher).

Briefly, a 9cm diameter filter paper disc was sprayed with antistatic cling spray (Static Guard, Proctor and Gamble, Cincinnati, OH), dust was weighed and applied to the disc to produce a consistent and even distribution, and the filter paper disc was enclosed in a 9cm Petri dish. The antistatic spray had no effect on survival of the bugs (data not shown). A 20µl spot (not exceeding 2cm diameter) of (E)-2-hexenal or (E)-2-octenal in acetone was applied to opposite sides of the filter paper and 2cm from the edge of the dish; applications were air-dried prior to adding the desiccant dust. Bugs were placed, 5 at a time, in the center of the filter paper disc and allowed to roam freely through the desiccant dust for 10min. This was conducted at 40% RH and 25°C, conditions consistent with human comfort standards (Reinikainen et al., 1992; Ferng and Lee, 2002). After a 10 min exposure in the Petri dish, bugs were removed, dust was removed using a stream of air, and each bug was then placed into a 1cc mesh-covered chamber so they could be monitored individually for determinations of water balance characteristics.

Harborage-evacuating experiments

To determine if these chemicals can be utilized to expel bed bugs from their off-host harborages, quiescent bed bugs were examined. A 20 x 40 cm plastic container without a cover (to allow pheromone dispersal), was divided into four
parallel sections of 10cm. The relative humidity was maintained at 33% throughout the experiments using saturated MgCl₂ (Winston and Bates, 1960). The first section remained empty, and the second was treated with Dri-die. The third remained empty, and fourth was the location for a 1 x 1 cm section of paper folded at 45° to offer a harborage for the bed bugs. Bed bugs (N =10, replicated five times) were introduced into the arena and quarantined until all ten bugs were quiescent under the harborage. After the bed bugs ceased moving, the desiccant was applied, followed by the alarm pheromone. Survival of the bed bugs was monitored until all ten bed bugs died or 40 days had elapsed for the first-instar nymphs and 80 days for the adult females. Concentrations of the chemicals applied and the type of desiccant were based on those most effective in the short-range experiments. Additionally, three more replicates with 15 individuals each were conducted after 24h to determine the water loss rates of first-instar nymphs and females.

Determination of water balance characteristics

Bugs were weighed using an electrobalance (CAHN; SD \pm 0.2µg precision and \pm 6µg accuracy at 1mg; Ventron Co., Cerritos, CA). Each bug was weighed individually, within less than 1 min, without enclosure or anesthesia; bugs were released from the aspirator tip and permitted to walk onto the weighing pan of the balance and were returned to their chambers. After being weighed, the bugs were placed at 0% RH (Drierite; Toolson, 1978) in a sealed 3000cc glass desiccator; the desiccator contained a perforated porcelain plate that provided a platform for the bugs above the Drierite located at the base of the desiccator. While 0% is not a relative humidity to which bed bugs are exposed in their natural environment, it is the only RH where there is no interference from the effects of passive sorption, i.e. the rate is not masked by sorption, as described by Wharton (1985). Bugs were weighed daily and each net transpiration rate was based on five consecutive mass measurements. Bugs were then placed in a 90°C (0% RH) drying oven and weighed until mass remained constant, which was defined as the dry mass (d). The dry mass was subtracted from each mass measurement to convert the values into water mass (m) values. The initial water mass was expressed as a proportion of the fresh mass x 100 to calculate the percentage body water content. To determine net transpiration rates (integumental plus respiratory water loss), each consecutive mass measurement was converted into a water mass value ($m_0 = initial$ water mass; $m_t = water$ mass at any time t) and fit to the equation $m_t = m_0 e^{-kt}$, which permits the net transpiration rate (-k) to be derived from the slope of a regression on a plot of $\ln m_t/m_0$ against time. This equation for net transpiration rate determination only applies if mass measurements are obtained at 0% RH because it is only under these conditions that water loss is exponential (Wharton, 1985). Survivorship was based on 50% mortality. Observations were used to verify the following criteria for death: unable to right and crawl, failure to respond to prodding or bright light.

Sample sizes and statistics

A total of 45 individuals (3 replicates of 15 bugs each) was used to determine each water balance characteristic. Each replicate was from a separate rearing batch of bugs. Data are presented as mean \pm SE. Comparisons were made using an analysis of variance (ANOVA), and percentage data were arcsin transformed prior to analysis (Sokal and Rohlf, 1995). Characteristics derived from regression lines were compared using a test for the equality of several slopes (Sokal and Rohlf, 1995). Survivorship estimates were compared using t-statistics.

Results

Body size of bed bugs

Fresh (initial) mass, water mass and percentage body water content were nearly identical for all first instar nymph: average of 0.13mg fresh mass, 0.037mg dry mass, 0.093mg water mass, and 71.54% body water content, with no significant differences between groups that were used in Dri-die and diatomaceous earth exposure experiments (Tables 5.2a and b; ANOVA; P > 0.05). For the females, the average fresh mass was 5.51mg, 1.82 mg dry mass, 3.69 mg water mass, and 66.97% body water content; there were no significant differences between groups in this study. In all cases, water mass was a positive correlate of dry mass, with $R \ge 0.94$ -0.96 (ANOVA; P < 0.001) for bugs that were exposed to Dri-die (Table 5.2a and b) and R ≥ 0.94 -0.98 (ANOVA; P < 0.001) for bugs that were exposed to diatomaceous earth (Table 5.3a and b).

Dri-die treatment in Petri dish assays

First instar nymphs and adult females exhibited a consistent exponential pattern of water loss at 0% RH, thus permitting the net transpiration rate to be derived from the slope of the line (Fig. 5.5a and b). All treatments involving a 10 minute

crawling exposure to Dri-die resulted in an increase in net transpiration rate when compared to controls that crawled on filter paper treated only with acetone (Fig. 5.5a and b). Exposure that combined Dri-die with [0.01M] (E)-2-hexenal:(E)-2-octenal blend resulted in a nearly 3-fold increase in the net transpiration rate (Fig. 5.5a and b). An approximate 2-fold increase in net transpiration rate was observed when [0.01M] (E)-2-hexenal or [0.01M] (E)-2-octenal was tested individually (Fig. 5.5a and b). Concentrations of [0.001M] (E)-2-hexenal or (E)-2-octenal or the (E)-2-hexenal:(E)-2-octenal blend had no effect on increasing the net transpiration rate of the bugs and produced results not significantly different from results obtained by exposure to only Dri-die (Table 5.2a and b, P > 0.05). [0.1M] (E)-2-hexenal, (E)-2-octenal or the (E)-2-hexenal:(E)-2-octenal blend increased the net transpiration rate, but the increase was not as pronounced as the effect at [0.01M] (Table 5.2). Consistently, the effect of the (E)-2-hexenal:(E)-2-octenal blend was greater than the effect of either component tested separately. Thus, bed bugs that were exposed to Dri-die in combination with a synthetic blend of alarm pheromone components lost water at a much faster rate than those treated with Dri-die alone.

Survivorship of 50% of first instar nymphs at 0% RH (25°C) was 4.1 ± 0.4 days for acetone-only controls (no Dri-die) and 2.6 ± 0.8 days when exposed to Dridie (t- statistics; P < 0.05). Survivorship dropped to 1.2 ± 0.4 day after exposure to Dri-die plus the [0.01M] (E)-2-hexenal:(E)-2-octenal blend, 3 days earlier than in the controls (t-tests; P < 0.05) and 2 days earlier (1.6 ± 0.7 days, t-tests; P < 0.05) for (E)-2-hexenal and 1.8 ± 0.9 days earlier for (E)-2-octenal alone (no significant difference between these two compounds; t-test; P > 0.05) when these components were tested individually. Females exposed to only Dri-die survived $17.0 \pm 1.1d$ at 0% RH; those exposed to either (E)-2-hexenal or (E)-2-octenal survived approximately 9d, and adult females treated with Dri-die and the chemical blend survived $6.5 \pm 1.9d$. The higher net transpiration rates for the bed bugs consistently translated into higher rates of mortality.

Diatomaceous earth treatment in Petri dish assays

A 10 minute exposure to diatomaceous earth had no appreciable effect on net transpiration rates of first instar nymphs or adult females (Tables 5.3a and b). But, net transpiration rates of the bugs increased 1.5-fold when diatomaceous earth was combined with (E)-2-hexenal or (E)-2-octenal and 2.0-fold when the (E)-2-hexenal:(E)-2-octenal blend was used at a concentration of 0.01M. At a lower concentration, [0.001M], no appreciable effect on net transpiration rate was noted for either the single compounds or the blend. Exposure to diatomaceous earth with [0.1M] (E)-2-hexenal, (E)-2-octenal, and the blend caused a slight, but significant, increase in net transpiration rate (Tables 5.3a and b). The synthetic alarm pheromone blend had a greater impact on increasing net transpiration rate than did the individual components at all concentrations (Tables 5.3a and b).

Net transpiration rates of bugs after exposure to Dri-die were higher than rates after exposure to diatomaceous earth (Tables 1a and b; ANOVA; P<0.05). This was reflected in longer survival in dry air (25°C) for diatomaceous earth treatments than for Dri-die treatments. Survival time of bed bugs treated with diatomaceous earth (4.3 ± 0.4 days) did not differ significantly from the acetone-only treated controls (3.7

 \pm 0.9 days, no significant difference; t-statistics; P > 0.05). With a pheromonal additive, survivorship dropped to 2.6 \pm 0.3 days with [0.01M] (E)-2-hexenal, 2.4 \pm 0.5 days with (E)-2-octenal (t-statistics; P > 0.05), and 1.9 \pm 0.7 days for the (E)-2-hexenal:(E)-2-octenal blend (Tables 2a and b; t-statistics; P < 0.05). Results were similar for adult females: diatomaceous earth only reducing survival in the presence of alarm pheromone components. Thus, Dri-die consistently generated a greater increase in the net transpiration rate than did diatomaceous earth, and the difference resulted in reduced survival time.

Termination of quiescence and departure from harborage

Based on the above results, only Dri-die and concentrations of alarm pheromone components or blends of 0.1M were evaluated for termination of quiescence and departure from their harborages. Our results suggest that application of alarm pheromone components caused the immediate dispersal of bed bugs from their harborages onto areas where Dri-die was present Application of the alarm pheromone resulted in a significant reduction, by at least 50%, in survival of firstinstar nymphs and adult females when used in combination with Dri-die (Fig. 5.6). All the bed bugs evacuated their harborage in less than 5 min. As before, application of the alarm pheromone resulted in a higher water loss rate than when Dri-die was used alone (Fig. 5.7), and this is likely to be the factor that reduced survival. We conclude that application of the alarm pheromones, (E)-2-hexenal and (E)-2-octenal, can be utilized to evoke dispersal of bed bugs from their harborages and, in combination with a desiccant, may enhance control.

Discussion

In response to [0.01M] (E)-2-hexenal and (E)-2-octenal, bed bugs displayed an excited, continuous searching behavior, crawling rapidly over the bottom of the Petri dish and failing to settle into clusters at the edge of the dish. Response to 0.1M was not as high in most cases, and may result from the complete saturation of pheromones, possibly leading to a lower response. Thus, these bed bugs exhibited a classic alarm response. Pheromones are often mixtures of compounds that are concentration and proportion specific (Blum, 1985), and this appears to be the case for the bed bug alarm pheromone. The alarm pheromone consists of two major active ingredients, (E)-2-hexenal and (E)-2-octenal, with the (E)-2-hexenal predominating in the mixture when it is released naturally by adults (Levinson et al., 1971; 1974). Consistent with the predominance of (E)-2-hexenal in the blend, the response to (E)-2-hexenal observed in this study was more pronounced than the response to (E)-2octenal, and the response to either component alone was lower than the response to the natural (E)-2-hexenal: (E)-2-octenal blend. The most useful application of the bed bug alarm pheromone may be to cause dispersal. Applying a desiccant such as Dridie and then using the alarm pheromone to evoke increased bed bug movement may be a useful technique for controlling bed bugs, including pesticide-resistant strains. It is important to note that even though the desiccating conditions in this study appear to be severe, bed bug responses would likely be similar, possibly because the bugs are adapted to dry human comfort standards (30-50% RH).

Species that can be effectively controlled by desiccant dusts include a variety of arthropod pests, but most noteably ticks (Allan and Patrican, 1994), cowpea weevils (Appel et al., 1999), cockroaches and silverfish (Faulde et al., 2006a,b), ants (Brinkman and Gardner, 2001) and numerous pests of grain (Akbar et al., 2004; Athanassiou et al., 2005). We now extend this list to include bed bugs, with Dri-die but not diatomaceous earth. The efficacy of diatomaceous earth appears to depend somewhat on the formulation; sometimes it works and sometimes it does not (Allan and Patrican, 1994). Resistance also appears to be an issue with diatomaceous earth (Korunic and Ormesher, 2000; Rigaux et al., 2001). Previous studies concluded that Dri-die seems to be superior to diatomaceous earths (Allan and Patrican, 1994; Appel et al., 1999), and that is what we observed in this study during short-term exposure. Two key points that may alter the effectiveness of Dri-die and DE are the duration of bed bug exposure and the residual effects. Indeed, future studies are needed to test these two aspects for *C. lectularius*. Even so, this would not change our interpretations that short term exposure to Dri-die, particularly in the presence of alarm pheromones, is more effective than DE for altering bed bug water balance characteristics. Lastly, how well the approach of using Dri-die with an alarm pheromone additive will work under large-scale field conditions, using human comfort standards, remains to be tested.

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A. First instar nymphs

Water balance characteristic:				
Treatment	<i>f</i> (mg)	<i>m</i> (mg)	%	NTR (%/h)
Acetone-only control	0.12 ± 0.05^a	0.09 ± 0.02^a	75.0 ± 1.8^{a}	0.461 ± 0.014^{a}
DD + Acetone	0.13 ± 0.04^{a}	0.09 ± 0.02^{a}	69.2 ± 2.2^a	$0.582 \pm 0.017^{b} \\$
DD + (E)-2-hexenal				
0.001M	0.12 ± 0.03^{a}	0.08 ± 0.02^{a}	66.7 ± 1.5^a	$0.563 \pm 0.017^{b} \\$
0.01M	0.15 ± 0.02^a	0.11 ± 0.02^a	73.3 ± 1.7^{a}	$0.913\pm0.023^{\text{d}}$
0.1M	0.13 ± 0.03^{a}	0.09 ± 0.01^a	69.2 ± 1.2^{a}	$0.624 \pm 0.013^{\circ}$
DD + (E)-2-octenal				
0.001M	0.13 ± 0.04^a	0.10 ± 0.01^a	76.9 ± 2.4^a	$0.592 \pm 0.014^{b} \\$
0.01M	0.11 ± 0.05^a	0.10 ± 0.03^a	71.4 ± 1.6^a	$0.845 \pm 0.014^{d} \\$
0.1M	0.14 ± 0.04^{a}	0.08 ± 0.02^{a}	72.7 ± 1.2^{a}	0.598 ± 0.018^{c}
DD + Blend				
0.001M	0.14 ± 0.04^a	0.10 ± 0.03^a	71.4 ± 1.7^a	$0.501 \pm 0.012^{b} \\$
0.01M	0.12 ± 0.02^a	0.08 ± 0.01^a	66.7 ± 2.0^a	1.289 ± 0.015^{e}
0.1M	0.15 ± 0.02^a	0.10 ± 0.01^a	66.7 ± 1.7^{a}	0.706 ± 0.021^{d}

B. Adult females

	Water balance characteristic:			
Treatment	<i>f</i> (mg)	<i>m</i> (mg)	%	NTR (%/h)
Acetone-only control	5.49 ± 0.05^a	3.62 ± 0.02^a	65.9 ± 1.8^{a}	0.104 ± 0.018^a
DD + Acetone	5.41 ± 0.04^a	3.59 ± 0.02^a	66.4 ± 2.2^a	$0.162 \pm 0.012^{b} \\$
DD + (E)-2-hexenal				
0.001M	5.43 ± 0.03^a	3.64 ± 0.02^a	67.0 ± 1.5^a	$0.171 \pm 0.017^{b} \\$
0.01M	5.55 ± 0.02^a	3.57 ± 0.02^a	64.3 ± 1.7^a	0.251 ± 0.023^{c}
0.1M	5.52 ± 0.03^a	3.60 ± 0.01^a	65.2 ± 1.2^{a}	0.184 ± 0.013^{b}
DD + (E)-2-octenal				
0.001M	5.54 ± 0.04^a	3.60 ± 0.01^a	65.0 ± 2.4^{a}	0.192 ± 0.014^{c}
0.01M	5.57 ± 0.05^a	3.66 ± 0.03^a	65.7 ± 1.6^{a}	0.231 ± 0.014^{c}
0.1M	5.61 ± 0.04^a	3.71 ± 0.02^a	66.1 ± 1.2^{a}	$0.179 \pm 0.011^{b} \\$
DD + Blend				
0.001M	5.53 ± 0.04^a	3.71 ± 0.03^a	67.1 ± 1.7^{a}	0.221 ± 0.012^{c}
0.01M	5.48 ± 0.02^{a}	3.58 ± 0.01^a	65.3 ± 2.0^{a}	0.411 ± 0.015^{d}
0.1M	5.41 ± 0.02^a	3.58 ± 0.01^a	66.2 ± 1.7^{a}	0.241 ± 0.021^{c}

Table 5.2. Comparison of net transpiration rates (integumental plus respiratory water loss) in (A) first instar nymphs and (B) female adults of *Cimex lectularius* after 10 min of crawling through Dri-die (label rate) with and without the addition of bed bug alarm pheromone. DD, Dri-die; f, fresh (initial) mass; m, initial water mass; %, initial percentage water content; NTR, net transpiration rate following treatment; Blend, 30:70 for larvae and 75:25 for adults, v/v (E)-2-hexenal: (E)-2-octenal. Data shown are mean \pm SE, N = 45. The same superscripts within a column denote no significant difference (ANOVA; P>0.05).

A. First instar nymphs

	Water balance c			
Treatment	<i>f</i> (mg)	<i>m</i> (mg)	%	NTR (%/h)
Acetone-only control	0.14 ± 0.03^a	0.11 ± 0.03^a	73.3 ± 2.3^a	0.395 ± 0.022^{a}
DE + Acetone	0.11 ± 0.02^a	0.08 ± 0.01^a	72.7 ± 2.1^a	0.421 ± 0.021^{a}
DE + (E)-2-hexenal				
0.001M	0.15 ± 0.04^a	0.09 ± 0.02^a	66.7 ± 1.3^{a}	0.441 ± 0.020^{a}
0.01M	0.12 ± 0.04^a	0.08 ± 0.02^a	75.0 ± 1.8^{a}	0.582 ± 0.016^{c}
0.1M	0.12 ± 0.05^a	0.08 ± 0.01^a	66.7 ± 1.2^{a}	$0.495 \pm 0.018^{b} \\$
DE + (E)-2-octenal				
0.001M	$0.14\pm0.04a$	$0.10\pm0.01a$	$71.4 \pm 2.0a$	$0.413 \pm 0.019a$
0.01M	0.13 ± 0.02^a	0.09 ± 0.02^a	69.2 ± 2.3^a	$0.574 \pm 0.012^{\rm c}$
0.1M	0.10 ± 0.03^a	0.07 ± 0.01^a	70.0 ± 1.5^a	0.479 ± 0.013^{b}
DE + Blend				
0.001M	0.11 ± 0.03^a	0.08 ± 0.02^a	72.7 ± 1.9^{a}	0.477 ± 0.020^{a}
0.01M	0.14 ± 0.02^a	0.11 ± 0.02^a	78.6 ± 1.8^a	0.796 ± 0.025^{d}
0.1M	0.15 ± 0.04^a	0.11 ± 0.03^{a}	73.3 ± 1.3^{a}	$0.517 \pm 0.011^{\circ}$

B. Adult females

	Water balance c	haracteristic:		
Treatment	<i>f</i> (mg)	<i>m</i> (mg)	%	NTR (%/h)
Acetone-only control	5.49 ± 0.15^a	3.70 ± 0.13^a	67.3 ± 3.5^a	0.104 ± 0.011^{a}
DE + Acetone	5.51 ± 0.17^a	3.76 ± 0.19^{a}	$68.2 \pm 1.5^{\rm a}$	0.109 ± 0.018^{a}
DE + (E)-2-hexenal				
0.001M	5.42 ± 0.21^a	3.66 ± 0.17^a	67.5 ± 3.1^a	0.114 ± 0.017^{a}
0.01M	5.58 ± 0.13^a	3.74 ± 0.11^a	67.0 ± 2.7^a	0.191 ± 0.015^{a}
0.1M	5.48 ± 0.19^{a}	3.71 ± 0.22^{a}	67.7 ± 2.2^a	0.121 ± 0.015^{a}
DE + (E)-2-octenal				
0.001M	5.62 ± 0.09^a	3.80 ± 0.18^{a}	67.6 ± 2.1^{a}	0.133 ± 0.024^{a}
0.01M	5.43 ± 0.15^a	3.60 ± 0.16^{a}	66.3 ± 2.6^a	0.186 ± 0.012^{b}
0.1M	5.61 ± 0.13^a	3.77 ± 0.09^{a}	67.2 ± 1.9^{a}	0.173 ± 0.023^{b}
DE + Blend				
0.001M	5.53 ± 0.14^{a}	3.69 ± 0.14^{a}	66.7 ± 3.7^a	0.133 ± 0.012^{a}
0.01M	5.59 ± 0.19^{a}	3.75 ± 0.12^{a}	67.1 ± 1.8^{a}	$0.263 \pm 0.012^{\circ}$
0.1M	5.64 ± 0.16^a	3.79 ± 0.20^a	67.2 ± 2.7^a	0.173 ± 0.012^{b}

Table 5.3. Comparison of net transpiration rates (integumental plus respiratory water loss) in (A) first instar nymphs and (B) adult females of *Cimex lectularius* after 10 min crawling through diatomaceous earth (label rate) with addition of bed bug alarm pheromone. DE, diatomaceous earth; f, fresh (initial) mass; m, initial water mass; %, initial percentage water content; NTR, net transpiration rate following treatment; Blend, 30:70 for larvae and 75:25 for adults, v/v (E)-2-hexenal: (E)-2-octenal. Data shown are mean \pm SE, N = 45. The same superscripts within a column denote no significant difference (ANOVA; P>0.05).



Figure 5.5. Proportion of water mass lost at 0% RH, 25°C by (A) first instar nymphs and (B) adult females of *Cimex lectularius* after 10 minute exposure to Dri-die (label rate) with and without alarm pheromone added. At 0% RH conditions, the slope of the regression through the points on the semilog plot is the net transpiration rate (integumental plus respiratory water loss). m_t , water mass at any time t; m_0 , initial water mass. Each point is the mean of 45 bugs and the SEs lie within the confines of symbols used on the graph. Data shown are for 0.01M of alarm pheromone, the concentration showing the greatest effect.



Figure 5.6. Survival of (A) first instar nymphs and (B) adult females of *Cimex lectularius* within a microhabitat to test Dri-die effectiveness with and without alarm pheromone components. Times denote when bed bug survival was measured. Each measurement represents the results form 50 individuals. Hexenal, (E)-2-hexenal; Octenal, (E)-2-octenal; Blend, combination of (E)-2-hexenal and (E)-2-octenal in a ratio of 30:70 for larvae and 75:25 for adults.



Figure 5.7. Water loss rates of (A) first instar nymphs and (B) adult females of *Cimex lectularius* after 24h in a microhabitat containing Dri-die. Each value is the mean \pm SE of 45 bugs.

Chapter 6: Habitat requirements of the seabird tick, Ixodes uriae (Acari: Ixodidae), from the Antarctic Peninsula in relation to water balance characteristics of eggs, nonfed and engorged stages

Abstract

The seabird tick *Ixodes uriae* is exposed to extreme environmental conditions during the off-host phase of its life cycle on the Antarctic Peninsula. To investigate how this tick resists desiccation, water requirements of each developmental stage were determined. Features of *I. uriae* water balance include a high percentage body water content, low dehydration tolerance limit, and a high water loss rate, which are characteristics that classify this tick as hydrophilic. Like other ticks, *I. uriae* relies on water vapor uptake as an unfed larva and enhanced water retention in the adult, while nymphs are intermediate and exploit both strategies. Stages that do not absorb water vapor, eggs, fed larvae and fed nymphs, rely on water conservation. Other noteworthy features include heat sensitivity that promotes water loss in eggs and unfed larvae, an inability to drink free water from droplets, and behavioral regulation of water loss by formation of clusters. We conclude that *I. uriae* is adapted for life in a moisture-rich environment, and this requirement is met by clustering in moist, hydrating, microhabitats under rocks and debris that contain moisture levels that are higher than the tick's critical equilibrium activity.

Introduction

Only a single tick species, *Ixodes uriae*, lives in Antarctica. On that continent, the tick feeds predominantly on penguins, but it is distributed at high latitudes in both hemispheres, presumably spread by migratory seabirds (>48 avian species recorded) that serve as its preferred hosts (Wilson, 1970). As a competent vector of *Borrelia* spirochetes to birds, *I. uriae* has epidemiological significance (Olsen et al., 1993; 1995). Physiologically, few experiments have probed *I. uriae* 's remarkable ability to survive in extreme cold and dry conditions. Those studies that have been conducted focus primarily on how this tick copes with extremely low temperature (Lee and Baust, 1982;1987). Interestingly, no physiological adaptations, i.e., cold tolerance or desiccation resistance (Lee and Baust, 1987; Dautel and Knülle, 1996), have been described that would make Antarctica a suitable habitat exclusively for *I. uriae*, but not other ticks.

Under field conditions, *I. uriae* takes approximately 3 years to develop from larva to adult, annually spending more than 11 months off the host, residing in aggregations underneath rocks and debris that sometimes reach thousands of individuals (Eveleigh and Threlfall, 1974). Each of these tick colonies consist of a mixture of ticks in all stages of development both before and after blood feeding, and are typically in close proximity to penguin rookeries. While clustered, these ticks remain relatively akinetic, huddling in direct contact with each other. They leave the aggregation in the protected, sheltered area only to feed, undoubtedly guided by bird host cues (kairomones; Sonenshine, 1991). After each bloodmeal, the ticks immediately return to their colony under the rocks where they molt, and wait until the bird hosts return the following season or, in the case of the adult female ticks, mate (mating takes place off-host in this tick), lay eggs and die (Sonenshine, 1991).

In this study, dehydration resistance of *I. uriae* was ascertained by determining standard water balance characteristics for each stage of the life cycle, from egg to adult. Dehydration tolerance was recorded as an indicator of the minimum amount of body water required for function. Water retention was assessed from water loss rate in relation to temperature. Clustering of this tick was evaluated as a method of water loss suppression. Free water drinking and water vapor absorption were examined as means to replenish losses and maintain their internal body water mass levels. Additionally, all developmental stages of the tick were compared to determine which periods in the life cycle were most vulnerable to water stress. In particular, we determined percentage body water content; dehydration tolerance limit; water loss rate; critical transition temperature (CTT), denoting the temperature where water loss increases abruptly; and critical equilibrium activity (CEA), the lowest amount of ambient moisture that is required for water vapor absorption to occur.

Materials and Methods

Tick collection, storage and weighing

Ixodes uriae were collected on the Antarctic Peninsula (64°04'S, 64°03'W) from Humble Island near Palmer Station, Anvers Island in January 2006, and all experiments were conducted at Palmer Station. Male and female adults, nymphs and

larvae were found grouped together under rocks, typically in sites near Adelie penguin (*Pygoscelis adeliae*) rookeries. Ticks were handled using soft-tipped forceps or an aspirator. In the experiments, eggs were used 7–10 days post-oviposition, larvae were used 10–14 days after ecdysis in the laboratory at 4°C, but the only age information we can provide for other stages is that they were acquired at the same time. Eggs were tested for viability by holding them in groups at 100% relative humidity (RH) at both 4 and 25°C for 1 month or until emergence. Experiments on engorged stages (larvae and nymphs) were conducted after they had ceased movement in preparation for molting, denoted by extension of the legs and failure by the tick to respond to stimuli (Kahl and Knülle, 1988). Temperature (4 and 25 \pm 1°C) and photoperiod (15:9 h light:dark) were controlled using environmental cabinets. Fed female adults were not analyzed because they die shortly after oviposition and adult males do not blood feed, thus larvae and nymphs were the only fed stages examined.

In the laboratory, ticks were held individually in 1 cc mesh-covered chambers placed on a perforated porcelain plate within a sealed glass desiccator (8,000 cc) that contained, at its base, a saturated salt solution to maintain RH. Each RH was controlled by saturated salt solutions containing an excess of solid salt; 33% RH (MgCl₂), 75% RH (NaCl), 85% RH (KCl) and 93% RH (KNO₃) or glycerol–distilled water mixtures of different concentrations (Johnson, 1940; Winston and Bates, 1960). Additionally, double-distilled water was used for 100% RH and CaSO₄ was used for 0% RH. Each RH was measured daily with a hygrometer (SD \pm 0.5% RH; Thomas Scientific, Philadelphia, PA) and varied less than 1% over the course of the

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experiments. An electrobalance (SD \pm 0.2 µg precision and \pm 0.6 µg accuracy, CAHN, Ventron Co., Cerritos, CA) was used to weigh the ticks without use of anesthesia.

Each specimen was taken from the desiccator, removed from its 1 cc enclosure with an aspirator, placed or allowed to crawl onto the weighing pan, weighed, picked up with an aspirator, placed back into the enclosure and then returned to the desiccator. This manipulation required approximately 1 min.

Measurement of water balance characteristics

Temperature used for basic observations was 25°C for comparison with previous studies of water balance (Hadley 1994) and 4°C as a temperature that is more representative of this tick's natural environment and for comparison with the cold hardiness literature (Lee and Baust ,1982; 1987; Worland and Block, 2003). For water balance purposes, activity units were used to define water movement into and out of the tick; a_v is the activity of water as a vapor ($a_v = \% RH/100$) and a_w is the activity of water as a liquid, which has been determined experimentally in terrestrial arthropods to be $0.99a_w$ based on mole fraction (Wharton 1985); thus, the activity of the tick's body water can be related directly to the water vapor in the surrounding atmosphere. Before experimentation, ticks were placed at $0.33a_v$ and monitored until loss of 4–6% body mass so that the ticks were physiologically standardized by removing any residual surface water and to minimize the effects of digestion, reproduction and defecation on mass changes; thus, mass measurements reflected only changes in the tick's body water levels (Arlian and Ekstrand 1975; Wharton 1985).

Water balance characteristics were determined based on Wharton (1985) with modifications by Benoit et al. (2005). In accordance with Wharton's (1985) general water balance equation (Eq. 1):

 $m = m_S - m_T$,

m is the water mass (amount of body water that is available for exchange with water vapor) and is influenced by water gain due to sorption (m_S) and water loss by transpiration (m_T). When $m_S > m_T$ there is net water gain, when $m_T > m_S$ there is net water loss, and when $m_S = m_T$ the tick is in water balance (no change in water mass).

The water mass (m) was calculated by taking the difference between the initial mass (i) and dry mass (d). After the initial mass was determined, dry mass was found by freezing (-70° C, 12h) and placing them at $0.00a_{v}$ at 65° C until reaching a constant mass. The body water content was found using Eq. 2:

percentage m = 100(i - d)/i

The maximum reduction of water mass a tick can tolerate before succumbing to dehydration-induced mortality was determined by exposure to $0.33a_v$, weighing them every 2 h. For nonfed stages this was based on when the tick failed to right itself and crawl ten body lengths, for eggs this was based on collapse of the chorion, lack of visible guanine accumulation internally and inability to hatch, and for fed stages it was the inability to emerge to the next developmental stage (Yoder and Spielman

1992; Yoder et al. 2006). To verify that individuals could not recover from dehydration, all specimens were placed at water saturation $(1.00a_v)$ for 24 h and again evaluated for survival. At the completion of the experiment, dry masses (d) were obtained so that mass measurements could be converted to water mass (m). The critical mass (m_C), used to assess dehydration tolerance, was defined as the mass below which the ticks could not be rescued by placing them at $1.00a_v$. This value was used to calculate the percentage change in mass (dehydration tolerance) according to Eq. 3, with i serving as the initial mass

percentage change in $m = 100(i - m_C)/i$

Water loss (m_T)

To prevent interference of adsorbed surface water from the atmosphere on mass changes, water loss rate (net transpiration rate = integumental plus respiratory water loss) was determined at $0.00a_v$ so that mass changes could be attributed solely to a reduction of the internal water pool; that is, under $0.00a_v$, m = $-m_T$ so water loss occurs with no gain because $m_S = 0$. In short, the ticks were weighed, held at $0.00a_v$, reweighed to find a change in mass six times and dried to a constant mass to obtain dry mass (d) so that mass changes reflected changes in body water (m), denoted m_t (water mass at any time t) as a proportion of initial water mass (m₀). These mass values were used to establish the rate of water loss (-kt) by plotting the ln (m_t/m_0) against time according to Eq. 4 of Wharton (1985):

with the rate derived from the slope and expressed as %/h. Additionally, water loss rates were expressed as mg/h. To determine the threshold of particularly rapid desiccation, water loss rate determinations (0.00a_v, following Eq. 4) were made for individual, isolated ticks and recorded at multiple temperatures. In some cases, a point when water loss begins to increase dramatically with temperature may occur and is denoted by the CTT. The CTT was determined using Arrhenius analysis based on the following equation:

 $\ln k = -E_a/RgasT + \ln A$

where k is the water loss rate, E_a is the energy of activation, R_{gas} is the gas constant, T is absolute temperature and A is the frequency factor. Living, rather than killed, ticks were used because they give the same CTT value (Davis, 1974; Yoder and Spielman, 1992; Yoder and Tank, 2006), while providing more physiologically relevant information.

To test for possible behavioral regulation on water conservation, group effects on water loss were similarly assessed by placing individuals in groups of 5 or 10 (Yoder and Knapp, 1999). Due to a shortage of field collected adults, only nymphs, larvae and eggs were used in this section of the experiment. The mobile stages were held in groups within a 2 cc mesh-covered chamber, and eggs were held directly in the wells of a porcelain plate. Water loss rates were determined at 0.00a_v at 25°C, following Eq. 4, for an isolated individual within the group. After mass determination the individual was returned to the group. To discern individuals to be weighed within the group, a spot of paint (Pactra, Van Nuys, CA) was applied with a single bristle of a soft camel's hair brush to the dorsal idiosoma; paint had no effect on mass changes (data not shown).

In all cases, weighing intervals for determining water loss rates were 1 h except at higher temperatures and for smaller immature stages where losses were excessive and necessitated the use of shorter time intervals so that the water loss rate could be derived from six consecutive mass measurements with the tick displaying regular ambulatory activity and prior to reaching its critical mass (m_C).

Water gain (m_S)

The threshold for active uptake of atmospheric water, defined as the CEA (Wharton 1985), was examined by monitoring tick water mass at different water vapor activities for 10 days. Conforming to standard practice, 0.85, 0.93, 0.98 and 1.00a_v were the water vapor activities used as benchmark values. Additional water vapor activities generated with glycerol–water mixtures were used to narrow the range to obtain a more precise approximation of the CEA by lowering the water activity by 0.01a_v until water mass was not maintained, testing for the lowest water vapor activity where the tick maintained its water mass (m). Below the CEA, water loss occurs by simple diffusion because the activity of the surrounding air is less than the 0.99a_w activity of the tick's body water. Thus, in our study the CEA represents the

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lowest water vapor activity where the tick was able to maintain its water mass (m) for a period of 10 days.

To test for free water drinking, ticks were held at $0.33a_v$ and monitored until 10–12% body mass was lost. Experiments were conducted as described (Yoder and Spielman, 1992; Kahl and Alidousti, 1997). Ticks were then placed, 10 at a time, into 9 cm i.d. petri dishes with 10–15 droplets of deionized (DI) water stained with 0.1% Evans blue dye and observed (40x) for 15 min of every hour for 24 h to examine their reactions to the droplets. Sizes of droplets varied from 5 to 20 µl. After this exposure, the ticks were removed, washed with DI water to remove residual dye, and placed at 0.33a_v for 2 h. Ticks were dissected in 1.0% NaCl to liberate any blue coloration from their digestive tract as confirmation of drinking.

Sample size and statistics

Each experiment involved at least 24 ticks or eggs. Each tick was monitored individually. Data are presented as a mean \pm SE and compared using an analysis of variance (ANOVA) with arcsin transformation in the case of percentages. A test for the equality of slopes of several regressions was used to compare characteristics derived from regression lines (Sokal and Rohlf 1995).

Results

Water content and loss at different stages

The water balance profiles for each life cycle stage of *I. uriae* are presented in Table 1. Among the nonfed stages, initial mass, dry mass, water mass and percentage

body water content increased throughout development, with eggs having the lowest percentage body water content and adults having the highest (ANOVA, P < 0.05). Females had a smaller body size than males and smaller water content, but there was no significant difference in percentage body water content between the two sexes (ANOVA, P > 0.05). As anticipated, fed stages were characterized by having increased water mass compared to the preceding nonfed stage, a likely consequence of blood feeding. Ticks within each stage were consistently the same size, and in all cases, water mass correlated positively with dry mass: $R^2 > 0.93$ for nonfed females, > 0.84 for nonfed males, > 0.91 for nonfed nymphs, > 0.87 for unfed larvae, > 0.88for fed larvae; > 0.85 for fed nymphs, and > 0.94 for eggs (ANOVA, P < 0.001). Corresponding water mass ratios (m/d) were similar among nonfed females (2.3), nonfed males (2.5), nonfed nymphs (2.3), fed larvae (2.3) and fed nymphs (2.4), but were lower for unfed larvae (1.9) and eggs (1.4), stages that have a lower percentage body water content as a consequence of having a greater dry mass. These observations imply that water balance characteristics are stage-specific.

Nonfed adult males and females had similar water loss rates, corresponding to a loss of 13–15% (0.71–0.86 mg) of their water content per day at 0.00a_v and 25°C, and 4% (0.21–0.25 mg) per day at $0.00a_v$ and 4°C (Table 1). These values are consistent with both adults water mass and surviving approximately 2 days at $0.00a_v$ at 25°C and 7 days at $0.00a_v$ at 4°C. Thus, survival estimates match dehydration tolerance given a corresponding rate of water loss.

Unfed larvae became irreversibly dehydrated after 8–10 h, and nonfed nymphs survived approximately 1 day at 0.00a_v, survival times that are consistent

with their respective water loss rates (Table 6.1). Eggs had a water loss rate of 2%/h (1.41 · 10–3 mg/h), amounting to a loss of almost 50% per day and failed to hatch after less than 1 day at $0.00a_v$. Consistently, at the ecologically relevant temperature of 4°C, water loss rates of all nonfed stages of *I. uriae* dropped by nearly one-third compared to the rates observed at 25°C (Table 6.1) which amounted to a 3–4-fold increase in survival time.

When analyzed as percentages of the initial water content, water loss also exhibits a size-rate relationship (y = -0.38x - 0.19, $R_2 > 0.99$ at 25°C; y = -0.42x - 0.42x1.41, $R_2 > 0.99$ at 4°C; Fig. 6.1), thus adults of this tick retain water more effectively than immature stages as a consequence of surface area to volume properties. Fed stages and eggs were not included in this size analysis because they differ so markedly from nonfed stages due to the fact that they are blood filled or contain inert heavy yolk in the case of eggs. Temperature produces an accelerated water loss rate, 25–30°C for unfed larvae and 30–35°C for nonfed nymphs, males and females as evidenced by curves denoting temperature effects on water loss rate (Fig. 6.2a). When examined by Arrhenius analysis (Fig. 6.2b–f), water loss rate increases correspondingly with increasing temperature and yields proportionate losses that are typical of standard Boltzmann temperature function (in all cases, $R^2 = 0.97$: ANOVA, P < 0.001). There is evidence of a CTT, corresponding to the temperature threshold of a particularly rapid water loss, at $30.1 \pm 1.9^{\circ}$ C for unfed larvae, $32.4 \pm 1.5^{\circ}$ C for nonfed nymphs, 37.8 ± 1.7 °C for nonfed males, and 37.9 ± 0.9 °C for nonfed females. Eggs did not have a CTT ($R^2 = 0.98$). Thus, larvae and eggs are more prone to temperature-induced desiccation stress than nymphs and adults.

The water loss rates for the fed stages were markedly lower in respect to their initial water content (0.41%/h for fed larvae and 0.21%/h for fed nymphs) when compared to unfed larvae (3%/h) and nonfed nymphs (1%/h) (Table 6.1; ANOVA, P < 0.05). The high water loss rates when shown as absolute values (mg/h) are typical of small, immature stages and unless you relate the water loss rates to the initial water content (%/h), no comparisons can be made between the water loss rates for each stage. By comparison, fed stages displayed lower water loss rates and were more resistant to desiccation, tolerating about 3% greater water loss (dehydration tolerance) and lost about 7x less water (water loss rate) than before it fed (ANOVA, P < 0.05; Table 6.1). Ranked statistically, taking into account water loss rate and dehydration tolerance and percentage water content (ANOVA, P < 0.05) with regard to ability to handle desiccation stress: fed stages > nonfed adults > nonfed nymphs > eggs > larvae, thus the larvae were the most sensitive to desiccation.

Group effect

Unfed larvae, nonfed nymphs and eggs in groups of 5 and 10 were more effective in preventing desiccation than isolated ticks (Table 6.2). For the unfed larvae and nonfed nymphs, the clusters formed were tightly packed and all individuals were in direct contact with each other, and upon re-introducing the marked tick to the cluster after mass determinations, this tick crawled back to the group. Eggs are laid as a mass. Each stage tested had a reduction of 10–15% in their water loss rates in clusters of only 5 individuals, and rates were further suppressed by nearly 65% for individuals in groups of 10, compared to the water loss rates of isolated individuals. Similar percentage reductions in the water loss rate also occurred when experiments were conducted at 4 and 30°C (data not shown), indicating that the clustering effect does not only occur as a result of high or low temperature stress. To diminish an effect of surface area to volume properties, eggs, unfed larvae and nonfed nymphs in groups had similar water mass (Table 6.2) to those used in experiments on individual ticks (Table 6.1; ANOVA, P > 0.05), implying that the difference that we note in water conservation can be attributed to clustering.

Water replenishment

Liquid uptake did not occur for any developmental stage as evidenced by the absence of blue stain in the digestive tract (Table 6.1). Unfed larvae, nonfed nymphs and adults encountered the water by chance, thus they did not appear to be attracted nor repelled by the droplets. At no point during our observations did a tick lower and insert its hypostome into the droplet and appear to drink, though some did appear to rest near the edge of the droplets on occasion. Fed larvae and fed nymphs did not react to the droplets because they were used during apolysis and no longer have the ability to move. However, nonfed stages (larvae, nymphs and adults) could uptake water vapor, and the CEA increased with progressive development (Table 6.1), with larvae being able to absorb water vapor from drier air (0.84–0.86a_v) than adults that absorb water vapor closer to saturation (‡0.89av); the CEA of nymphs fell in between (0.86–0.88a_v). Eggs, fed larvae and fed nymphs could not utilize water vapor as evidenced by net water mass decreases at 0.98a_v (1 atmosphere lower than activity of the body water, 0.99a_w) of 0.20%/day by eggs, 0.08%/day by fed larvae and

0.04%/day by fed nymphs. We conclude that active water vapor uptake occurs in those stages that are not modified for water retention.

Discussion

We anticipated that *I. uriae* would display either a low water loss rate, low percentage body water content or high tolerance for dehydration because these water conservation features typically coincide with tolerance to low temperature (Danks, 2000). To the contrary, *I. uriae* is characterized by high water loss rates, classifying it as hydrophilic with regard to water balance, which implies that the emphasis for this tick is on water gain rather than water retention and desiccation-hardiness. To counter large body water losses, *I. uriae* seeks cool, moist reprieves under rocks, consistent with its hydrophilic nature, and these results in the formation of clusters. Importantly the moisture level under the rocks is greater than the CEA of the tick. In fact, what these data show are a remarkable similarity in water balance characteristics between *I*. *uriae* and other kinds of *Ixodes* ticks, suggesting that the occurrence of *I. uriae* on Antarctica is not directly related to a unique water balance characteristic, but instead is probably dictated by the presence of seabird colonies and attributes of cold tolerance. The propensity of *I. uriae* for moisture and absence of major water conservation features suggests that Antarctica is an ideal environment for *I. uriae* because it is cold, which enables the tick to control its rate of water loss. This is supplemented behaviorally by a water-conserving group effect as a result of aggregation formation. These ticks survive because they are cold tolerant (Lee and

Baust, 1987), but from a water balance perspective, the low temperature is critical for maintaining water balance.

On the islands near Palmer Station, the ground temperature remains relatively stable below 4°C throughout major portions of the year, especially under the rocks where *I. uriae* overwinters, indicating that cold exposure will not be debilitating to the populations (Lee and Baust, 1987). During the summer, the temperature will surpass 20°C only on a few occasions, and the moisture content near the tick colonies is rather high, averaging >0.96a_v under the rocks (field data 2006), allowing *I. uriae* to maintain its water balance after feeding and molting. These moisture conditions are greater than the CEA of the tick (0.84–0.86a_v for larvae, 0.86–0.88a_v for nymphs and 0.89–0.92a_v for adults) and thus are sufficiently high to permit water vapor absorption and maintain water balance; that is, the microhabitat under the rocks exceeds the CEA of the tick. Thus, given the importance of water gain for *I. uriae* because it is hydrophilic, it seems reasonable to suggest that the moisture-rich microhabitat under the rocks is selected to satisfy an absolute moisture requirement, and this results in the formation of clusters.

Clustering has the side benefit of reducing water loss rates of individual members. The result is a nearly two-fold decrease in the amount of water lost which makes a major contribution to the internal body water pool. Reduced rates of water loss occurred in tick clusters of five individuals, and further reduction occurred when group size was increased to ten (water loss rate dropped by nearly 30% when compared to the water loss rate observed for individuals). Whether the water loss rate continues to drop as group size increases, or reaches a threshold, is not known;

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neither is the mechanism. Conceivably, the cluster is a site of localized high water vapor activity generated by water loss of the members of the group producing a humidified boundary layer (Yoder et al., 1992) that perhaps can be used by nearby ticks. Behavioral regulation of water loss by group effects have been reported for other ticks (Dermacentor variabilis, Amblyomma americanum; Yoder and Knapp, 1999), but only for larvae, the stage regarded as most sensitive to desiccation (Knülle, 1966). Because nymphs and eggs of *I. uriae* have a group effect as well, the clustering is not solely a larval response to stress, rather a behavioral mechanism employed by multiple stages of this tick. In our recent field observations and those reported by Lee and Baust (1987), we typically observe clusters of hundreds of this tick indicating that water loss reduction by the formation of aggregations also occurs in nature and is not a laboratory artifact. The clustering effect of egg masses is especially important for *I. uriae* due to their high water loss rates, particularly if temperatures are high. Clustering offers the additional benefit of bringing males and females together for mating (plus pheromonal cues; Sonenshine, 1991), such that these moisture-rich sites (preferred by fed females; Sonenshine, 1991) under rocks are sites for oviposition, development and hatching.

Slowing down the water loss rate is the key survival element for *I. uriae*. Like many ticks, percentage body water content of *I. uriae* (70–75%) is relatively high (~70%; mean water content of most arthropods; Hadley, 1994), thus to function properly they require a high body water content, only about 1/3 of which can be lost before reaching a lethal level of dehydration. Several aspects of *I. uriae* enable water loss suppression. (1) Stable cold temperature, averaging 4°C, has an appreciable

impact on restricting water loss and keeping the water loss rates low. (2) Possession of a high $CTT > 30^{\circ}C$ for *I. uriae* largely safeguards this tick from experiencing any kind of abrupt, rapid lethal water loss. (3) Selection of moisture-rich sites at or above the CEA of *I. uriae* under the rocks, which has the advantage of exposing ticks to more hydrating atmospheres than dehydrating ones in addition to maintaining water balance. (4) Water loss is reduced behaviorally by group effects. Interestingly, eggs of *I. uriae* are nearly as prone to desiccation as the larvae, even though the egg has a lower water loss rate and a higher dehydration tolerance than larvae; the lack of a water uptake mechanism and the inability to move to more favorable conditions increases the likelihood of the eggs becoming irreversibly dehydrated. This suggests that both the eggs and larvae will dictate moisture requirements that may limit the expansion of *I. uriae*, particularly in relation to increasing temperature.

Our classification of *I. uriae* as hydrophilic is based mainly on its water loss rate in comparison to other species (Hadley, 1994). Water loss rates match moisture requirements for life in a particular environment; species that are dry-adapted have low water loss rates and species that are wet-adapted have high water loss rates (Hadley, 1994). Comparative water loss rates at 0.00a_v, 25°C, conditions that offer the greatest potential for comparisons, of nonfed adult females are: 0.67%/h for *I. scapularis* (Yoder and Spielman, 1992) and 0.28%/h for *A. americanum* (Sigal, 1990), two species that are classified as having a hydrophilic distribution (Hair and Bowman, 1986), 0.21%/h for *D. variabilis* (Yoder et al., 2004b), which has a distribution described as ubiquitous (Drummond, 1998), 0.15%/h for *Rhipicephalus sanguineus* (Yoder et al., 2006), a xerophilic species that displays a preference for warm and dry

climates (Heath, 1979; Demma et al., 2005), and 0.08%/h for the extreme xerophilic, desert-adapted *Hyalomma dromedarii* (Hafez et al., 1970). This places *I. uriae* (0.54%/h) more toward the hydrophilic end of the spectrum. Indeed, a hydrophilic classification is common for species of *Ixodes* (Lees, 1946; Kahl and Alidousti, 1997; Kahl and Knülle, 1988; Yoder and Spielman, 1992), and this agrees with their distribution in moisture-rich environments that are typically near the coast (Drummond, 1998).

As in other ticks, the capacity to uptake water vapor is specific to certain stages of the life cycle (Knülle and Rudolph, 1982; Needham and Teel, 1986). Larvae of *I. uriae* can use water vapor at a_v's as low as 0.84–0.86a_v, which corresponds to CEA values for larvae of A. americanum, A. cajennense, D. andersoni and D. variabilis (Knülle, 1966), Haemaphysalis leporispalustris (Camin and Drenner, 1978) and I. scapularis (Yoder and Spielman, 1992). The moisture requirement for adults, however, is greater, as demonstrated by CEA values that are closer to saturation: 0.89–0.92a_v in *I. uriae* and 0.92–0.96a_v for *I. canisuga*, *I. hexagonus* and *I. ricinus* (Lees, 1946) and A. americanum (Sigal, 1990). Nymphal CEA values are 0.86– $0.88a_v$ for *I. uriae*, which are close to that $(0.87-0.89a_v)$ for *I. scapularis* (Yoder and Spielman, 1992) and *I. ricinus* (Kahl and Knülle, 1988), and are intermediate between the CEA's of larvae and adults (Knülle and Rudolph, 1982; Yoder and Benoit, 2003). There are typically more hydrating atmospheres for larvae (CEA is lower) as a tradeoff for its high water loss rates and more dehydrating atmospheres for an adult (CEA close to saturation) to prevent over hydration due to its lower water loss rate; nymphs are intermediate and represent the shift in priority from water gain as a larva to water

retention as an adult (Yoder et al., 2006). Water vapor absorption does not occur in eggs (Teel, 1984; Yoder et al., 2004a) as this stage is uniquely modified for water conservation by having low water content (due to presence of inert, heavy yolk) and high tolerance for dehydration (Heath, 1979; Hinton, 1981). Engorged stages of *I. uriae*, like engorged stages of *I. holocyclus* and *I. ricinus* (Heath, 1981; Knülle and Rudolph, 1982; Kahl and Knülle, 1988) and *A. americanum* (Yoder et al., 1997), favor water retention rather than uptake and lose their ability to absorb water vapor from the air.

Ixodes uriae was incapable of drinking liquid water to maintain its off-host moisture levels, as also noted in two other Ixodes species, I. scapularis (Yoder and Spielman, 1992) and *I. ricinus* (Lees, 1946; Kahl and Alidousti, 1997), as well as other ticks A. cajennense (Knülle 1966) and D. nuttalli (Kahl and Alidousti, 1997). Ticks that can imbibe free water include *B. microplus*, *B. decoloratus*, *R. evertsi*, *R.* appendiculatus, A. americanum (summarized by Kahl and Alidousti, 1997) and R. sanguineus (Yoder et al., 2006). In all of these cases, the ticks were observed to approach dye-stained droplets of water, insert their hypostome into the droplet and drink the colored liquid. Evidence of dye can be seen filling the gut diverticula and the color tracer is liberated upon dissection. Failure of *I. uriae* to drink free water is thus a feature shared among many, but not all, tick species. Based on free water drinking, there does not appear to be any consistent ecological, phylogenetic or stage connection that would distinguish those species that can drink free water from those species that cannot. The fact that *I. uriae* does not drink directly from droplets of water is not unusual for *Ixodes*, but our preliminary mass change and behavioral data
suggest that they may replenish high body water losses by absorbing water vapor from the humidity in the vicinity of the droplet, as observed in *I. ricinus* (Kahl and Alidousti, 1997).

This study on *I. uriae* also addresses a crucial issue involving the rapid water loss that occurs in response to rising temperature, a point known as the CTT. The CTT is sometimes, but inappropriately, used as an indicator of temperature tolerance to predict the potential of a species to spread into new geographic regions. The CTT for larvae of I. uriae is 30° C, a value that is within the $30-35^{\circ}$ C range for larvae of A. americanum, A. maculatum, D. variabilis, I. scapularis and R. sanguineus (Yoder and Tank, 2006). A higher CTT of 38°C is observed in adults of *I. uriae*, a value that is within the 35–40°C range for adults of *I. ricinus*, *I. canisuga*, *I. hexagonus* and *A.* americanum (Lees, 1946). I. uriae is like A. americanum in that there is no CTT in the egg (Yoder et al., 2004a). Few reported CTT values are available for nymphs, but they tend to fall close to or within the range of larval values rather than the range for adults (Lees, 1946; Yoder and Spielman, 1992). There is no doubt that ixodid ticks experience an abrupt, rapid water loss as the temperature rises (Lees, 1946; Yoder and Tank, 2006). In most ticks, the CTT lies beyond the biological temperatures preferred by the species, suggesting that the CTT is of negligible importance (Needham and Teel, 1986). Indeed, the CTT values of *I. uriae* are far higher than temperatures that this tick naturally experiences on Antarctica, except possibly while feeding. Interestingly, the CTT for *I. uriae* is within the ranges of the CTT's of larvae and adults of other tick species that represent an array of thermal habitats, suggesting that the CTT of ticks cannot be used for ecological interpretations

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Characteristics	Eggs	Larvae		Nymphs		Males	Females
		Unfed	Fed	Nonfed	Fed		
Water pool Initial mass (mg)	0.121 ± 0.010	0.093 ± 0.003	1.282 ± 0.092	0.888 ± 0.061	11.57 ± 0.41	8.64 ± 0.13	6.83 ± 0.09
Dry mass (mg)	0.051 ± 0.037	0.032 ± 0.005	0.386 ± 0.074	0.269 ± 0.031	3.48 ± 0.35	2.46 ± 0.16	2.04 ± 0.11
Water mass (mg)	0.070 ± 0.029	0.061 ± 0.006	0.894 ± 0.011	0.611 ± 0.021	8.39 ± 0.24	6.18 ± 0.09	4.79 ± 0.06
Water content (%)	57.85 ± 1.21	65.60 ± 0.64	69.73 ± 1.62	69.43 ± 0.91	72.51 ± 1.72	71.56 ± 0.54	70.13 ± 0.72
Water loss Loss tolerance (%)	33.4 ± 2.5	23.2 ± 1.02	26.4 ± 0.9	25.2 ± 0.8	28.5 ± 1.5	28.7 ± 0.9	27.1 ± 1.5
WLR (%/h)(Fig. 2) 4°C 25°C	0.52 ± 0.06 2.01 ± 0.04	1.04 ± 0.09 3.05 ± 0.03	0.09 ± 0.01 0.41 ± 0.03	0.43 ± 0.03 1.41 ± 0.05	0.07 ± 0.02 0.21 ± 0.03	0.17 ± 0.01 0.58 ± 0.02	0.18 ± 0.03 0.62 ± 0.02
WLR (mg/h) 4°C 25°C CTT (°C)	$\begin{array}{c} 3.64 \pm 0.42 \times 10^{-4} \\ 1.41 \pm 0.03 \times 10^{-3} \end{array}$	$\begin{array}{c} 6.34 \pm 0.55 \times 10^{-4} \\ 1.86 \pm 0.09 \times 10^{-3} \\ 30.1 \pm 1.9 \end{array}$	$\begin{array}{c} 8.05 \pm 0.89 \times 10^{-4} \\ 3.66 \pm 0.03 \times 10^{-4} \\ \mathrm{ND} \end{array}$	$\begin{array}{l} 2.51 \pm 0.18 \times 10^{-3} \\ 8.62 \pm 0.31 \times 10^{-3} \\ 32.5 \pm 1.5 \end{array}$	$\begin{array}{c} 5.87 \pm 1.67 \times 10^{-3} \\ 1.76 \pm 0.25 \times 10^{-2} \\ \text{ND} \end{array}$	$\begin{array}{c} 1.05 \pm 0.07 \times 10^{-2} \\ 3.58 \pm 0.12 \times 10^{-2} \\ 37.8 \pm 1.7 \end{array}$	$\begin{array}{l} 8.62 \pm 1.14 \times 10^{-3} \\ 2.96 \pm 0.10 \times 10^{-2} \\ 37.9 \pm 0.9 \end{array}$
Water gain Liquid water uptake CEA (av) (Fig. 5)	- 20.99	- 0.84-0.86	- 20.99	- 0.86-0.88	- 20.99	- 0.89-0.90	- 0.89-0.92
Each value represents	the mean ± SE of at le	ast 24 individuals					
Loss tolerance, percent activity of water in the	age water loss at the c vapor phase = %RH/	ritical mass; WLR, wa 10; -, does not occur;]	ter loss rate at 0.00av ND, not determined	and 25°C; CEA, critic	al equilibrium activity,	where water vapor al	sorption begins; a_v ,

Table 6.1. Comparison of water balance characteristics of *Ixodes uriae* throughout development

Group size	N	Water mass (mg)			Water loss rate (%/h)		
		Eggs	Larvae	Nymphs	Eggs	Larvae	Nymphs
1 5	30 10	0.070 ± 0.008^{a} 0.068 ± 0.013^{a}	0.061 ± 0.0011^{a} 0.061 ± 0.0015^{a}	0.611 ± 0.021^{a} 0.607 ± 0.049^{a}	2.01 ± 0.024^{a} 1.69 ± 0.041^{b}	3.05 ± 0.025^{a} 2.72 ± 0.060^{b}	1.41 ± 0.05^{a} 1.21 ± 0.07^{b}
10	10	0.073 ± 0.020^{a}	0.060 ± 0.0016^{a}	0.601 ± 0.090^{a}	$1.32 \pm 0.061^{\circ}$	$2.01 \pm 0.020^{\circ}$	$0.99 \pm 0.05^{\circ}$

Each value represents the mean \pm SE. Data followed by the same superscript are not statistically different (ANOVA, P > 0.05)

Table 6.2. Effects of clustering at 25°C on the water loss rate of eggs, unfed larvae and nonfed nymphs of the tick, *Ixodes uriae*.



Fig. 6.1. Correlation between size of each developmental stage and water loss rate of *Ixodes uriae* at 4 (open squares) and 25°C (closed squares). Each point represents the water loss rates from at least 24 individuals. (L larva, N nymph, M male adult, F female adult)



Fig. 6.2. Temperature effect on ability to retain water (water loss rate) by *Ixodes uriae* at different stages of development (a closed circle larvae, open square eggs, open circle nymphs, closed square females, closed diamond males). Same data plotted on an Arrhenius plot (b–f). Water loss rates were determined at 0.00 a_v from 24 individuals and are presented as mean ± SE in Fig. A

7. Conclusions

This study determined dehydration resistance for three species of mosquitoes *(Aedes aegypti, Anopheles gambiae,* and *Culex pipiens)*, bed bugs (*Cimex lectularius*), and the seabird tick (*Ixodes uriae*). This was accomplished by performing standard water balance studies for all five species, examining the role of heat shock proteins in mosquitoes using RNAi, and examining how pheromones alter water balance. Additionally, this study determined the effects of multiple dehydration bouts on the physiology of mosquitoes.

I. Water balance of mosquitoes featuring the role of two heat shock proteins.

1. Fully hydrated females of all three species contained nearly the same amount of water (66-68%), but water loss rates differed among the species, with *A. aegypti* having the lowest water loss rate (2.6%/h), followed by *C.pipiens* (3.3%/h), and *A. gambiae* (5.1%/h).

2. In all three species water could be replaced only by drinking water (or blood).

3. *Aedes aegypti* and *C. pipiens* tolerated a loss of 36% of their body water, but *A. gambiae* was more vulnerable to water loss, tolerating a loss of only 29% of its body water.

4. Dehydration elicited expression of *hsp70* in all three species, but only *C*. *pipiens* continued to express this transcript during rehydration. *Hsp90* was constitutively expressed, and expression levels remained fairly constant during dehydration and rehydration, except expression was not noted during rehydration of *C. pipiens*.

5. Injection of dsRNA to knock down expression of *hsp70* and *hsp90* in *A*. *aegypti* did not alter water content or water loss rates, but the dehydration tolerance was lower. Instead of surviving a 36% water loss, females were able to survive only a 28% water loss in response to RNAi directed against *hsp70* and a 26% water loss when RNAi was directed against *hsp70*.
6. These results indicate a critical function for these Hsps in mosquito dehydration tolerance.

II. Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*

 Although their percentage water content was lower, diapausing females contained both higher initial and dry masses than nondiapausing individuals.
 Both nondiapausing and diapausing females tolerated a loss of up to 40% of their water mass before dying, but diapausing female *C. pipiens* reached this point after a longer period due to their lower rate of water loss. Males, which do not overwinter in diapause, showed no differences in their water balance characteristics when reared under diapausing or nondiapausing conditions. Likewise, no changes were noted in the water balance of pupae, indicating that diapause-related changes do not occur prior to adult eclosion
 This mosquito does not replenish internal water stores by generating metabolic water or by absorbing vapor from the atmosphere, but instead relies on drinking liquid water (or blood feeding in the case of nondiapausing females).

5. The critical transition temperature, a point where water loss increases rapidly with temperature, was the highest for females, then males, then pupae, but was not influenced by the diapause program. Females in diapause did not utilize common polyols (glycerol, trehalose and sorbitol) to retain water, but instead the presence of twice the amount of cuticular hydrocarbons in diapausing compared with nondiapausing females suggests that the deposition of hydrocarbons contribute to the reduced rates of water loss.

6. The laboratory results were also verified in field-collected specimens: mosquitoes in the late fall and winter had a lower percentage water content and water loss rate, higher initial mass, dry mass and more cuticular hydrocarbons than individuals collected during the summer.

7. Thus, the major features of diapause that contribute to the suppression of water loss are the large size of diapausing females (reduction of surface area

to volume ratio lowers cuticular water loss), their low metabolic rate and the deposition of extra cuticular hydrocarbons.

III. Influence of chronic versus acute dehydration stress on the physiology of the northern house mosquito, *Culex pipiens*

1. After six dehydration/rehydration bouts, mosquitoes provided access to sugar during the rehydration period had considerably higher survival than those only allowed to rehydrate, and survival was similar to mosquitoes of the same age that were not dehydrated.

2. After each bout, there was a reduction in the total dry mass of the mosquitoes that were not provided sugar during the rehydration periods between the bouts of dehydration. Dry mass reduction is likely due to the utilization of carbohydrate, glycogen and lipid reserves, and these reductions in nutritional reserves lead to decreased survival after multiple bouts of dehydration/rehydration.

3. The results indicate that diapausing mosquitoes experience a continual decline in lipid reserves with each bout of dehydration. These reduced metabolic reserves correlate with reduced survival time, indicating that diapausing females may not have adequate fat reserves for overwintering if exposed to multiple bouts of dehydration.

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4. Egg production for nondiapausing and post-diapause *C. pipiens* was reduced after multiple bouts of dehydration.

5. Overall, multiple dehydration bouts reduce the metabolic reserves of mosquitoes, likely due to the cost of responding to dehydration stress. The reduced nutritional reserves and survival can be alleviated by providing mosquitoes access to sugar during rehydration between dehydration bouts.

IV. Water balance of the bed bug, Cimex lectularius

1. This species is characterized by a low net transpiration rate averaging < 0.2%/h, high tolerance for dehydration (30–40% loss in body water), and an impermeable cuticle as indicated by a high critical transition temperature (CTT) in the 35–40°C range, implying that this insect is adapted for desiccation-hardiness. The capacity of adults to survive for 2 weeks at $0.00a_v$ ($a_v = \%$ RH/100) with no access to food or water exemplifies this trait. 2. In contrast to more mature stages, first-instar nymphs contain more water, lose water at a faster rate, experience abrupt water loss at a lower temperature, and survive less time in dry air, suggesting that this stage is the most sensitive to water stress.

3. This insect relies on blood to replenish water stores; none of the stages examined have the capacity to absorb water vapor (critical equilibrium activity, $CEA > 0.99a_v$), and they drank only sparingly when offered free water.

4. As the bed bugs progress through their development, they gradually reduce their water requirements while increasing their desiccation resistance. Surviving water stress is considerably enhanced behaviorally by quiescence, characterized by prolonged periods of inactivity, and by the formation of clusters that generate a water-conserving group effect.

5. With Dri-die alone, the water loss was 21% higher than in untreated controls, and water loss increased nearly 2x with (E)-2-hexenal and (E)-2-octenal, and 3x with the (E)-2-hexenal: (E)-2-octenal blend. This shortened survival of first instar nymphs from 4d to 1d, with a similar reduction noted in adult females.

6. Diatomaceous earth was effective only if supplemented with pheromone, resulting in a 50% increase in water loss over controls with the (E)-2-hexenal:(E)-2-octenal blend, and a survival decrease from 4d to 2d in first instar nymphs

7. Based on observations in a small microhabitat, the addition of alarm pheromone components prompted bed bugs to leave their protective harborages and to move through the desiccant, improving the use of desiccants for control. 8. We concluded that that the effectiveness of the desiccants can be further enhanced by incorporation of alarm pheromone. Presumably, the addition of alarm pheromone elevates excited crawling activity, thereby promoting cuticular damage that increases water loss.

V. Habitat requirements of the seabird tick, *Ixodes uriae*, from the Antarctic Peninsula in relation to water balance characteristics of eggs, nonfed and engorged stages.

1. Features of *I. uriae* water balance include a high percentage body water content, low dehydration tolerance limit, and a high water loss rate, which are characteristics that classify this tick as hydrophilic.

2. Like other ticks, *I. uriae* relies on water vapor uptake as an unfed larva and enhanced water retention in the adult, while nymphs are intermediate and exploit both strategies. Stages that do not absorb water vapor, eggs, fed larvae and fed nymphs, rely on water conservation.

3. Other noteworthy features include heat sensitivity that promotes water loss in eggs and unfed larvae, an inability to drink free water from droplets, and behavioral regulation of water loss by formation of clusters.

4. I conclude that *I. uriae* is adapted for life in a moisture-rich environment, and this requirement is met by clustering in moist, hydrating, microhabitats

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under rocks and debris that contain moisture levels that are higher than the tick's critical equilibrium activity.

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