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

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Summary

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 cuticular hydrocarbons in diapausing compared with nondiapausing females suggests that the deposition of hydrocarbons contribute 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.

Key words: mosquito, water balance, diapause, water loss rates, *Culex pipiens*.

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 4–5-week-old adult females that fed on a chicken and were allowed to oviposit in de-chlorinated tapwater. Larvae were held at a density of approximately 250 individuals in a 18 cm×25 cm×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 long-day 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 $CaSO_4$ 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), $MgCl_2$ (33% RH), $NaNO_2$ (65% RH), NaCl (75% RH), KCl (85% RH), KNO_3 (93% RH) and K_2SO_4 (98% RH). A hygrometer (s.d.±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. ±0.2 μg; accuracy, ±6 μg, 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):

$$\%M_w = 100\times(M_i–M_d) / M_i . \quad (1)$$

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:

$$\%M_w = 100\times(M_t–M_0) / M_0 , \quad (2)$$

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 ($-k_t$) was expressed as a loss rate in % h^{-1}:

$$M_t = M_0 \times e^{-k_t} . \quad (3)$$

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:

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

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 μl droplets of deionized water stained with 0.1% Evans Blue dye. The drops were placed on a 100 mm×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 40× 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 100× magnification.

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 l^{-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 l^{-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 μl 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×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 N_2, 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 N_2. 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 ± 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 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 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 1; ANOVA, P<0.05) than in those

Table 1. *Comparison of the water requirements of pupae, males and females of Culex pipiens reared under diapausing and nondiapausing conditions*

	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.

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 1). Additionally, pretreatments of –20°C for 2 min, CO_2 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. 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 non-diapausing individuals (Fig. 2). Temperature increases had nearly identical effects on the water loss rates of diapausing and nondiapausing individuals, based on the CTT values observed (Table 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 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). 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

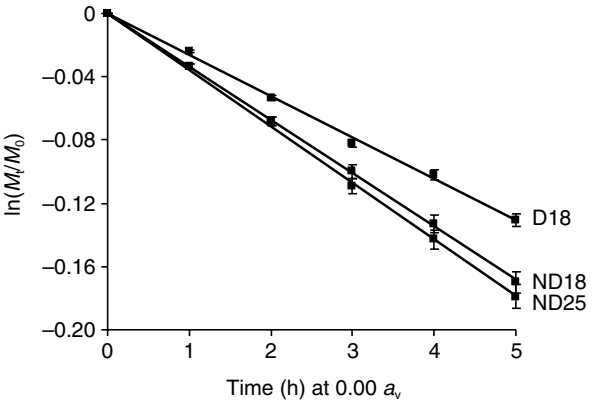

Fig. 1. Net water loss rates in females of *Culex pipiens* reared under diapausing (D18) and nondiapausing conditions (ND25; ND18) at 0.00 a_v. The slope of the regression through the points represents the water loss in % h^{-1}. M_t is the mass at any time t and M_0 is the initial water mass. Values 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.

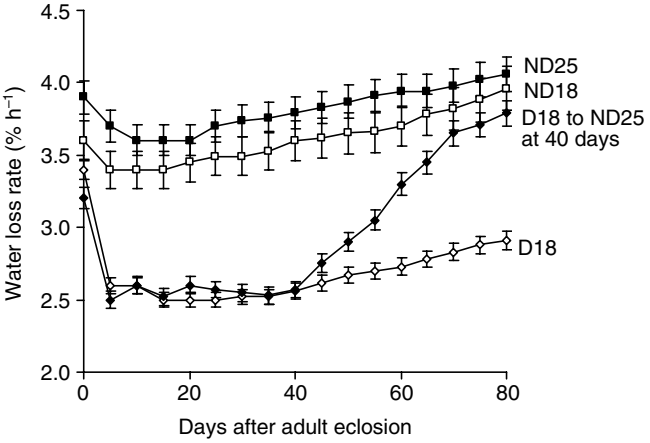

Fig. 2. Water loss rates in females of *Culex pipiens* over a prolonged period. Each point represents water loss determined as in Fig. 1. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes 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.

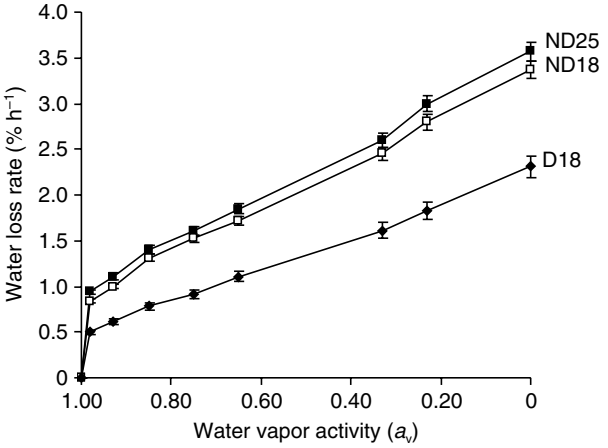

Fig. 3. Water vapor exchange at different vapor activities in females of *Culex pipiens*. For each point, the vapor exchange was determined as in Fig. 1. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing photoperiod (9 h:15 h, L:D) at 18°C.

between 1.00 a_v and 0.98 a_v (Fig. 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 (40× 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. 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. 4). The CTT was the same for winter- and summer-acclimated mosquitoes (40.2±0.9°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. 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. 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

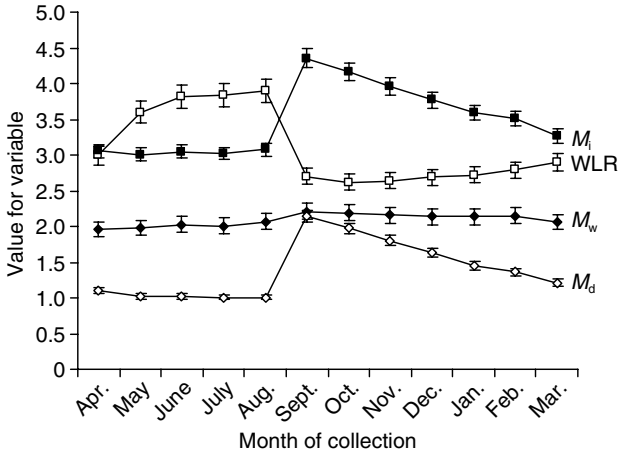

Fig. 4. Water balance characteristics of *Culex pipiens* females collected at monthly intervals from the field in Columbus, Ohio. WLR, water loss rate (% h^{-1}); M_d, dry mass (mg); M_w, water mass (mg); M_i, initial mass (mg). Values are means ± s.e.m. of 30 mosquitoes.

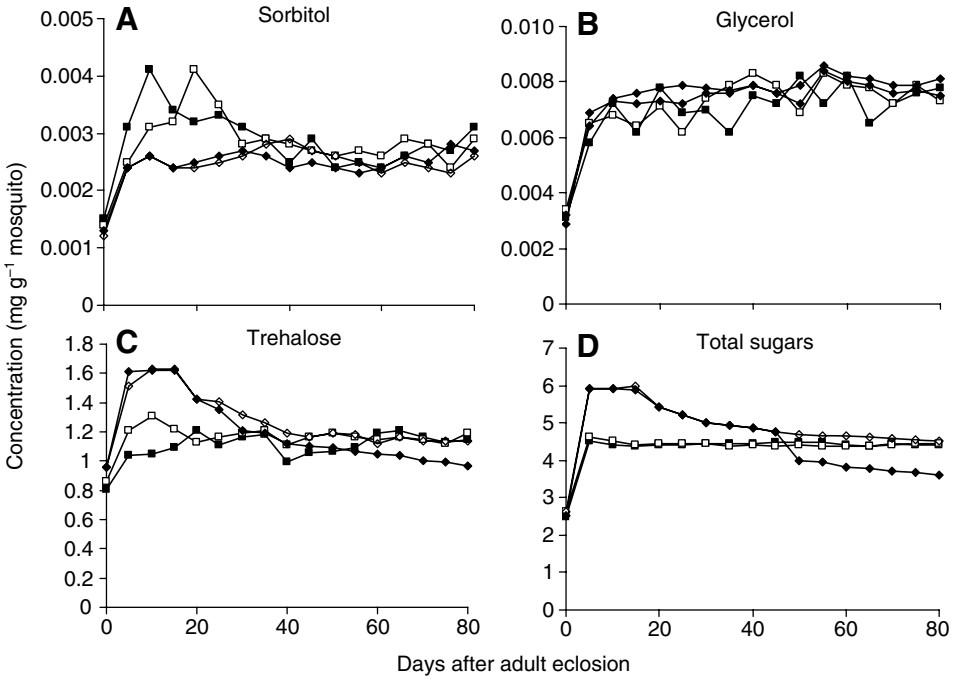

Fig. 5. Polyol and sugar content of nondiapausing and diapausing adult females of *Culex pipiens*. (A) Sorbitol; (B) glycerol; (C) trehalose; (D) total sugars. Closed squares, ND25 mosquitoes, reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; open squares, ND18 mosquitoes, reared under 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 ± s.e.m. of 10 replicates of five individuals each. All error bars are smaller than the symbols.

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 either glycerol, sorbitol or trehalose in samples collected in November 2005, February 2006 and June 2006 (Table 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. 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.

Table 2. *Amounts of polyols, sugars and cuticular lipids in field-collected populations of Culex pipiens collected from Columbus, Ohio, USA*

	Date of collection		
Concentration	Nov. 2005	Feb. 2006	July 2006
Polyols (ng g^{-1} mosquito)			
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} mosquito)			
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/mosquito)			
Hydrocarbons	440±65	412±62	220±42[a]
Polar components	1310±120	1160± 215	1310±192

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

Fat utilization

Nondiapausing mosquitoes, reared at 18 or 25°C, utilized lipids much faster than diapausing mosquitoes (Fig. 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

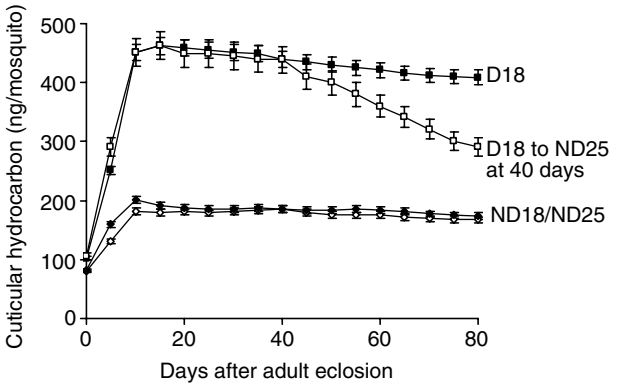

Fig. 6. Amount of cuticular hydrocarbons extracted from nondiapausing and diapausing females of *Culex pipiens*. ND25, mosquitoes reared under a nondiapausing photoperiod (15 h:9 h, L:D) at 25°C; ND18, mosquitoes reared under a nondiapausing photoperiod at 18°C; D18, mosquitoes reared under a diapausing 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 ± s.e.m. of 10 replicates of five mosquitoes each.

Fig. 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 ± s.e.m. of 10 replicates of five mosquitoes each.

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_v the respiration rate possibly decreases and thereafter remains constant, allowing water loss to increase linearly with further decreases in vapor activity.

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 μg 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 laboratory-reared mosquitoes by 40–50% reduced mosquitoes to a size similar to the field-collected mosquitoes (J. B. Benoit, personal observation), but the percentage water content was not different between the two groups, thus indicating that the field-collected 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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