

FUNCTION OF THE URNULAE IN PROTECTING THE RED VELVET MITE, *BALAUSTIUM* SP., AGAINST WATER LOSS AND IN ENHANCING ITS ACTIVITY AT HIGH TEMPERATURES

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ABSTRACT - Features of *Balaustium* sp. include resistance to intense heat and desiccation, affinity for hot surfaces in bright light, abundance in semi-arid/arid biotopes, and a large pair of secretory glands called urnulae with no known function (defense secretion excepted). Here we show that the urnulae secrete a waterproofing barrier that reduces the mite's cuticular permeability to water. Exposure to white light was used to stimulate release of the secretion; the urnulae protruded and exuded streams of red fluid at the tip of this structure that covered the entire body. Results showed that mites coated with urnulae secretion lost water at approximately half the rate of mites that did not secrete. Similarly, urnulae secretion coated mites demonstrated an increase in water-tightness of the cuticle reflected by a 9 °C elevation in temperature threshold for water loss on an evaporation curve, increasing their optimal temperature tolerance for survival (lethal permeability temperature, LPT). Results also show a 10 kJ/mol drop in activation energy (E_a) for water loss, representative of a substantial cuticular modification, and a decrease in Arrhenius frequency steric factor A , indicating an overall decrease in body water losses. The absence of a critical transition temperature (CTT), however, reveals that urnulae secretion coating functions to resist a phase change as the temperature rises, permitting the mites to cope with high temperature without succumbing to water and heat stress, by inhibiting cuticular breakdown.

Key words - Desiccation resistance, critical transition temperature (CTT), velvet mite, *Balaustium*, Ohio, USA.

INTRODUCTION

A red velvet mite, *Balaustium* sp. (Parasitengona: Erythraeidae) in Ohio, can be found abundantly in gardens and on wall surfaces in early summer. They are easily recognized by their bright red color and rapidly duck in and out of crevices in brick walls, concrete foundations, rocks, tree trunks, plants and leaf litter, searching for food (scale insects, soft-bodied arthropods, phytophagous mites and eggs) or sites for oviposition (Putnam, 1970; Childers and Rock, 1980; Welbourn and Jennings, 1991). In Australia, *Balaustium* mites are pests in grain (Halliday, 2001) and some species (although not the one studied here) bite humans (Newell, 1963). In nature, they have an affinity for hot surfaces, predominately in areas that are exposed fully to sunlight, and are successful in semi-arid to arid biotopes (Wohltmann, 1998; Yoder *et al.*, 2006a, 2007a). Despite its minute body size, one of the most remarkable features of this soft-bodied terrestrial

mite is its ability to tolerate high temperatures of up to 50 °C for prolonged periods while resisting desiccation (Yoder *et al.*, 2007a). In addition to being suited for a dry environment (xerophilic water balance in ecologic classification), water conservation of these erythraeid mites has been suggested to be associated with their ability to seal off body openings (Wohltmann, 1998). There is no evidence for high temperature conditioning in *Balaustium* sp. (Yoder *et al.*, 2007a). No other features have been described for *Balaustium* sp. that link desiccation-resistance and heat tolerance to their ability to function on hot surfaces.

When disturbed or exposed to intense heat or bright light, *Balaustium* sp. secretes a stream of fluid that spreads over the body surface facilitated by numerous setae (Yoder *et al.*, 2006b). The secretory sources of this fluid are the urnulae, a pair of large glandular openings positioned directly behind the eyes; these structures are present in the adult and nymphs, but not in the larva

(Newell, 1963). When stimulated, the urnulae protrude from the dorsal surface as collar-like tubercles and exude streams of a red-colored fluid from the tip that evaporates over the mite's body surface (Yoder *et al.*, 2006b). This secretion has defensive (allomonal) and alarm functions (one of the active ingredients has been chemically identified as neryl formate, a common mite alarm pheromone; Yoder *et al.*, 2007b). Physically, the secretion strongly resembles hemolymph (mite blood) with coagulating/defensive properties as a novel form of reflex bleeding, conceivably related to aposematism (Yoder *et al.*, 2006b). The mite's capacity to function properly in the environment is dependent on their ability to maintain adequate levels of body water (= water balance; Wharton, 1985), largely dictated by how fast it loses water (Hadley, 1994). Necessary maintenance of body water is particularly problematic for these mites because their minute body size makes them especially prone to drying (large surface area to volume ratio). Here, we examine adult *Balaustium* sp. mites, comparing their net transpiration rates (integumental and respiratory water loss), and passive water loss (integumental water loss), in response to rising temperature (as in calculation of critical transition temperature, CTT; Gibbs, 2002; Yoder *et al.*, 2005b) of mites that have secreted from their urnulae with mites that have not secreted. The primary goal of this study is to determine whether this hemolymph-based secretion from the urnulae in *Balaustium* sp. may have a waterproofing function.

MATERIALS AND METHODS

Mites - Mite taxonomists have told us that our Ohio *Balaustium* is a new species and until it is described we have been advised to refer to it as *Balaustium* sp. (voucher specimens are stored in the Acarology Laboratory, Museum of Biological Diversity, The Ohio State University, Columbus, OH, under specimen lot number OSAL 013113). *Balaustium* sp. were field collected from landscape, brick walls and concrete foundations (Springfield, OH) and represent a three year study conducted during the peak season (when thriving populations consists of thousands of individuals). Sample collections were performed daily with the use of an aspirator and occurred during the following dates: (1) 20 - 30 June 2006, (2) 2 - 15 June 2007, and (3) 15 - 20 May 2008, mainly between 0700 - 1100 hours. Studies used only female adults that were distinguished by an adult female *Balaustium* as figured by Newell (1963) and Gabrys (2000). Live mites were used for a particular experiment within 30 minutes of collection. Killed mites were likewise used in a freshly killed condition and were obtained by freezing (-20 °C overnight) and thawing to room temperature (25 °C) or killed in a closed system of HCN vapor. Each mite was stored in an individual 1 cc mesh-covered chamber, moni-

tored singly, and placed onto a perforated porcelain plate in a 2000 cc sealed glass desiccator. Mites were of unknown age, but were collected approximately at the same time of day, and results were reproducible among replicates.

Mass determinations were obtained using an electrobalance (precision of $SD \pm 0.2 \mu\text{g}$ and an accuracy of $\pm 6 \mu\text{g}$ at 1 mg; CAHN, Ventron Co., CA). Each mite was individually transferred with an aspirator directly to the weighing pan of the balance (with no enclosures) and then returned to test conditions in less than 1 minute. The only relative humidity used for this investigation was 0% RH (still air conditions; Drierite; CaSO_4 ; Toolson, 1978; measured with a hygrometer $SD \pm 2\%$ RH; Thomas Scientific, Philadelphia, PA). Under 0% RH conditions, no water is available for sorption and water loss is exponential, permitting the rate of loss to be derived from the slope of a regression as described by Wharton (1985). Thus, mass measurements are not masked by passive water gains that occur at other moisture conditions. Temperature was controlled using programmable calibrated cabinets ($< \pm 0.5 \text{ }^\circ\text{C}$). At the end of each experiment, the mites were placed into a 90 °C drying oven over CaSO_4 and dried to completion. Mites were then weighed daily until mass remained constant, and this was designated as the dry mass (*d*).

Water content - The amount of water available for exchange is defined as the water mass (*m*). Water mass was calculated by subtracting the dry mass from each interval mass measurement. Percentage body water content was calculated from the initial (fresh) mass (*f*) as defined by the following equation: percentage $m = 100\% (f - d)/f$ (Wharton, 1985).

Water loss rate - The water loss rate of living mites at 0% RH conditions is called the net transpiration rate (integumental plus respiratory water loss). However, the water loss rate of killed mites is called the passive water loss rate (integumental water loss only) because the respiratory component has been removed. Due to this integumental phenomenon, the examination of critical transition temperature requires the use of freshly killed mites (Seethaler *et al.*, 1979; Wharton, 1985; Gibbs, 2002; Yoder *et al.*, 2005b). Calculation of the water loss rate (whether living or killed mites) was based on five consecutive mass measurements (varying time intervals dependant of temperature) of individual mites at 0% RH. Each mass measurement was converted to its corresponding water mass (*m*) value. To determine the rate of water loss expressed in %/h, the water mass values were fit to the following equation: $m_t = m_0 e^{-kt}$ or $\ln(m_t/m_0) = -kt$, where m_t is the water mass at any time *t*, m_0 is the initial water mass and $-kt$ is the rate of water loss (Wharton, 1985).

Analysis of water loss rate (critical transition temperature, CTT) - Net transpiration rate (living mites)

was determined at the standard temperature of 25 °C to permit comparison with other water balance literature (Hadley, 1994). Passive water loss rate (killed mites) was determined at 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C to examine temperature effects such as the critical transition temperature. Passive water loss rate as a function of temperature was analyzed in two ways. The two methods of analysis allowed for determination of the lethal permeability temperature (LPT) and the critical transition temperature (CTT). Lethal permeability temperature is defined as the temperature at which water loss increases abruptly and corresponds to the maximum temperature that can be tolerated for survival (Yoder *et al.*, 2007a). The critical transition temperature is the temperature at which cuticular lipids undergo a phase change resulting in a dramatic water loss (Hadley, 1994; Rourke and Gibbs, 1999). The first method was analyzed using an evaporation curve, or linear plot (passive water loss rate vs. temperature), in accordance with interpretations by Lees (1946, 1947), Beament (1959) and Yoder *et al.* (2007). This assessment of data determines the LPT. The second method for analysis was by an Arrhenius plot (ln passive water loss rate vs. reciprocal absolute temperature) fitted to the Arrhenius equation: $k = Ae^{-E_a/(R_{gas}T)}$ or $\ln k = -E_a/(R_{gas}T) + \ln A$, where E_a is the activation energy, k is the passive water loss rate, A is the frequency (steric) factor, R is the universal gas constant (8.314J/mol/K to give units for E_a in J/mol), and T is absolute temperature. The critical transition temperature was then determined by identifying a change in activation energy (derived from the slope = $-E_a/R_{gas}$ on the Arrhenius plot) in accordance with interpretations by Gibbs (2002).

Urnulae secretion, experimental design and statistics - To stimulate the secretion of the urnulae, mites (live) were exposed to intense flashes of bright white light and prodded with a forceps while anchored to a piece of double stick tape mounted on the stage of a stereomicroscope (40x). Upon secretion, the urnulae were observed protruding and releasing a red fluid that covered the mite body giving it a residual sheen (Yoder *et al.*, 2006b). After the mites had secreted, they were immediately used for determination of the net transpiration rate or killed for the critical transition temperature study.

Data are mean \pm SE. Each water loss rate represents a total of 75 mites at each temperature. The total group of 75 mites was comprised of three replicates (25 per replicate, each from a different sampling date). It is important to note that a comparison was performed between two different treatment groups of mites; one group of mites that had not secreted from the urnulae (control) and another group of mites that were induced to secrete by exposure to bright light and disturbance by prodding. The 'secreted' and 'not-secreted' (control) comparison was conducted on three different groups of mites: living (25 °C only for net transpiration rate determination), killed (10 - 70 °C for passive water loss rate determination; CTT) by freeze-

thaw method and killed (10 - 70 °C for passive water loss rate determination; CTT) by the HCN exposure method. An analysis of variance (ANOVA) was used to compare data (the arcsin transformation was used for percentages). Water loss rates were compared using Sokal and Rohlf's (1995) test for the equality of slopes of several regressions. We emphasize that the same group of mites was not used for determination of all of the water balance characteristics.

RESULTS

Body size and water content - Values for initial mass, dry mass, water mass and percentage body water content are shown in Table 1. No significant differences were noted with regard to these water content characteristics between mites that did not secrete and mites that had secreted (ANOVA; $P > 0.05$). In all cases, the water mass was a positive correlate of the dry mass: for mites used in the net transpiration rate experiment, $R \geq 0.94$ for mites that had secreted and $R \geq 0.89$ for mites that did not secrete, and for mites used in the critical transition temperature experiment, $R \geq 0.91$ for mites that had secreted and $R \geq 0.95$ for mites that did not secrete (ANOVA; $P < 0.001$). Water mass to dry mass ratio were similar among the groups of mites, averaging 2.8 for mites having secreted and 2.7 for mites that had not secreted (ANOVA; $P > 0.05$). All mites were about the same size and shape in the experiment. These similarities with regard to water content and size characteristics between treatment groups imply that mites are physiologically synchronized and surface area to volume effects are negligible, which compensates for their unknown age. We conclude that stimulating the secretion of fluid from the urnulae in mites does not alter body water content.

Net transpiration rate (living mites) - Integumental and respiratory water loss (net transpiration rate; NTR) followed a typical exponential pattern ($R = 1.00$; ANOVA; $P < 0.001$) at 0% RH and 25 °C. These results reflect steady, proportionate body water losses (i.e., no interference from passive adsorbed water that occurs at higher relative humidities) such that the rate was calculated from the slope of the regression line (Fig. 1). Mites that had secreted from the urnulae had a net transpiration rate of 1.33% water/h. This was approximately 2x slower than the 2.47% water/h net transpiration rate for mites that had not secreted (Table 1; ANOVA; $P < 0.05$). To confirm our net transpiration rate determinations, each treatment group of mites was observed for survival at 0% RH, 25 °C. Mites that had not secreted survived (50% of mites) for 16 - 21 hours under these conditions. However, as the differences in net transpiration rate suggested, the mites that had secreted and had the lower net transpiration rate survived for 27 - 32 hours (each $N = 100$ mites). Thus, adult female mites retain water more effectively

Table 1. Impact of urnulae secretion on water content, net transpiration rate (NTR; using live mites) and critical transition temperature (CTT; using killed mites) in adult female *Balaustium* sp. *f.*, fresh (initial) mass; *d*, dry mass; *m*, water mass; %, percentage body water; LPT, lethal permeability temperature; and E_a , activation energy. Mites killed by freeze/thaw (shown) and HCN vapor yielded nearly identical results. Data shown are mean \pm SE.

Characteristic	NTR experiment (N=75)		CTT experiment (N=525)	
	Secreted	Not secreted	Secreted	Not secreted
Water content				
Initial mass, <i>f</i> (mg)	0.117 \pm 0.023	0.124 \pm 0.019	0.128 \pm 0.016	0.121 \pm 0.028
Dry mass, <i>d</i> (mg)	0.030 \pm 0.012	0.035 \pm 0.014	0.035 \pm 0.013	0.032 \pm 0.009
Water mass, <i>m</i> (mg)	0.087 \pm 0.017	0.089 \pm 0.018	0.093 \pm 0.024	0.089 \pm 0.019
<i>m/d</i> (mg)	2.9	2.5	2.7	2.8
Water content (%)	74.36 \pm 1.8	71.77 \pm 1.3	72.66 \pm 1.5	73.55 \pm 1.4
Water loss				
NTR (%/h; Fig. 1)	1.33 \pm 0.17	2.47 \pm 0.12	-	-
LPT ($^{\circ}$ C; Fig. 2A)	-	-	58-60	50-52
CTT ($^{\circ}$ C; Fig. 2B)	-	-	Not detected	Not detected
E_a (kJ/mol; Fig. 2B)	-	-	34.42 \pm 0.7	47.03 \pm 0.3

(net transpiration rates are lower) following secretion from the urnulae than mites that have not secreted.

Critical transition temperature (killed mites) - Integumental water loss increased progressively with temperature exhibiting a typical curve shape, indicative of

an exponential function observed on a linear plot (Fig. 2A). The inflection in the curvature indicates where passive water loss increases rapidly. For mites that had secreted, this inflection in the curve occurred between 58 - 60 $^{\circ}$ C whereas control mites that had not secreted exhib-

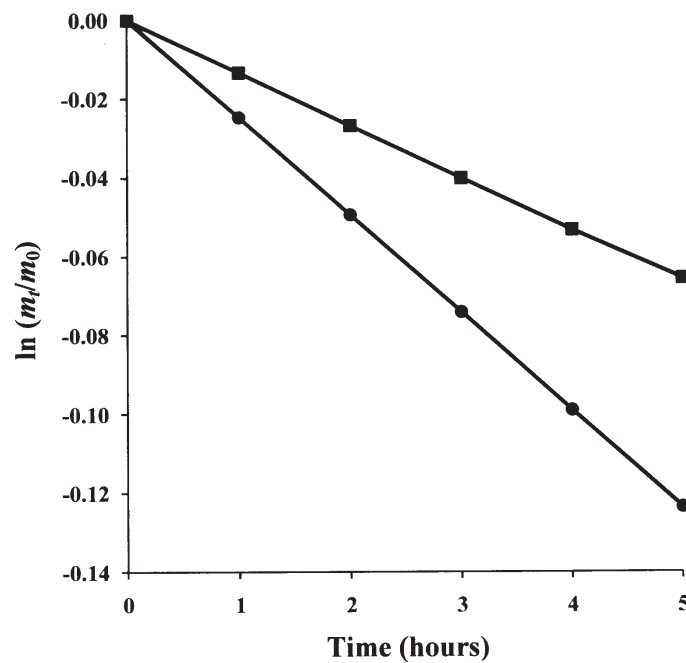


Fig. 1. Influence of secretion of urnulae on the net transpiration rate (living mites) of female *Balaustium* sp. based on the proportion of water mass (*m*) lost at 0% RH, 25 $^{\circ}$ C, conditions where water loss is exponential permitting net transpiration rate to be derived from the slope of the regression line. Square symbols, mites that had been induced to secrete; circle symbols, mites that did not secrete; m_t , water mass at time *t*; m_0 , initial water mass. Error bars lie within confines of symbols used on the graph, and each point represents mean \pm SE of 75 mites.

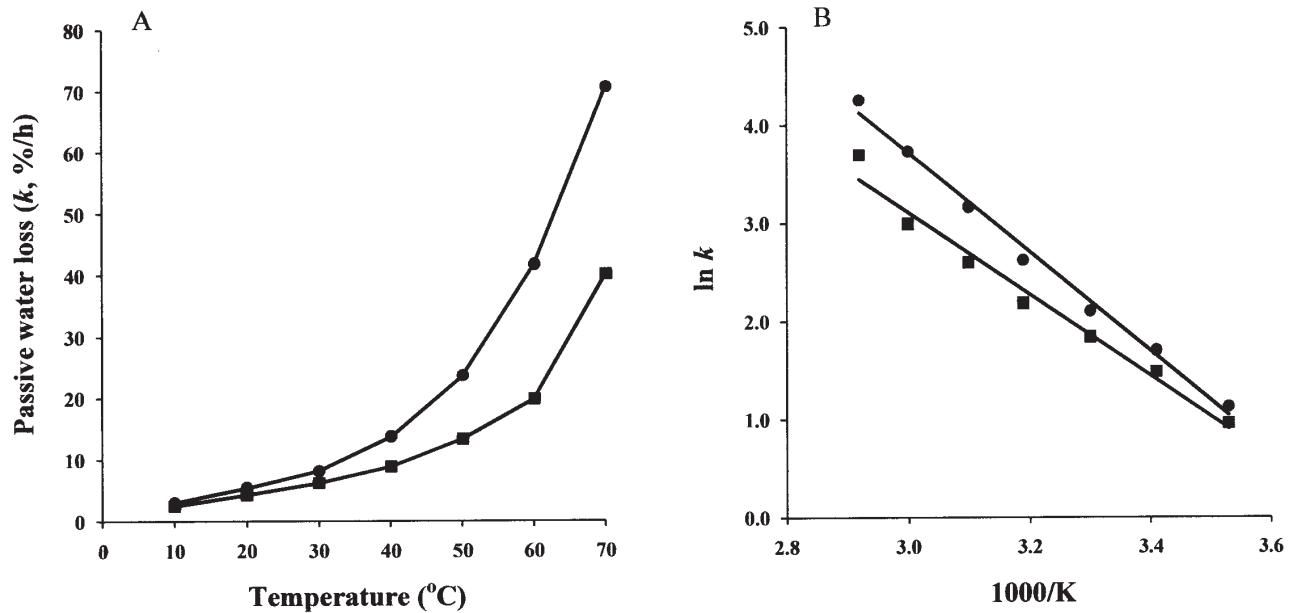


Fig. 2. Effect of temperature on passive water loss rate (killed mites) of adult female *Balaustium* sp. A, linear plot showing lethal permeability temperature (LPT) denoted by inflection on the curve; B, Arrhenius plot showing lack of critical transition temperature (CTT) because the slope of the line, corresponding to activation energy ($-E_a/R_{\text{gas}}$), does not change. Square symbols, mites that had been induced to secrete; circle symbols, mites that did not secrete. Freezer-killed mites (shown) yielded similar results as mites killed with HCN vapor. Each point is mean \pm SE (error bars lie within graph symbols) of 75 mites.

ited an abrupt increase in passive water loss at a lower temperature range, between 50 - 52 °C (Table 1; Fig. 2A). Similar results were obtained whether the mites were started at 70 °C and dropped down from one temperature to the next ('ramp down') or whether the mites were started at 10°C and moved up ('ramp up'; data not shown). As expected, water loss was 2 - 4x higher for killed mites than for living mites (Figs. 2A and 1, respectively). These results emphasize the importance of life processes (respiratory control) on regulating water loss rate. On the Arrhenius plot shown in Figure 2B, urnulae secretion resulted in a lower activation energy (E_a) for water loss: E_a 's of 47 kJ/mol ($y = -5061.5x$, $R = 1.00$; Fig. 2B) for mites before secretion versus 34 kJ/mol ($y = -4175.1x$, $R = 0.99$; Fig. 2B) for mites after having secreted (Table 1; ANOVA; $P < 0.05$). The frequency (steric) factor A also experienced a decline in mites as a result of having secreted, as evidenced by a decrease in y-intercept on the Arrhenius plot (Fig. 2B). There was no change in activation energy (continuous slope over a broad 10 - 70 °C temperature range; standard Boltzmann temperature function) for both mites that had secreted and mites that had not secreted, indicating that no critical transition temperature (CTT) was detected (Fig. 2B). We conclude that the secretion of the urnulae in female adult

Balaustium sp. functions as a deterrent to cuticular water loss.

DISCUSSION

Evidence indicates that the secretion from the urnulae (induced by a disturbance of intense bright light) provides extra waterproofing to adult female *Balaustium* sp. by making the cuticle more water-tight. This reduced cuticular permeability allows for the important maintenance of adequate levels of body water in order to function properly. Presumably, the ability of this fluid to fill pores and congeal while spreading over the body surface serves as a means to seal off body openings, minimize water stress, and characterize the dry adaptation of erythraeid mites as proposed by Wholtmann (1998). Evidence further suggests that this secretion also elevates the mite's resistance to high temperature by increasing the lethal permeability temperature. Therefore, the elaboration of this secretion, in part, accounts for the impressive capacity of this mite to function regularly on extremely hot surfaces in full sunlight.

The urnulae are leaky structures, emitting pulses of secretion when the mite is exposed to flashes of intense white light and they release large amounts of secretion

with longer durations of light exposure; thus, this secretion is delivered frugally, which is also consistent with its defensive/alarm function (Blum, 1985). The amount of secretory release is linked to the intensity, length and duration of the stimulus, which necessarily implies that the extent of waterproofing to avoid desiccation depends upon the brightness (heat) exposure. Because of the activation of secretion by urnulae in response to mechanical stimulus, it also seems reasonable to suggest that this secretion functions as a repair mechanism to prevent excessive water loss suffered from cuticular abrasion while rapidly crawling through soil, leaf litter and organic debris. One of the most important properties of this secretion is that it owes its waterproofing attributes to the fact that it resists thermal breakdown even at high temperature, as evidenced by lack of a critical transition temperature (CTT).

Several features of the Arrhenius plot display the reduced cuticular permeability to water as a result of urnulae secretion. The emphasis is on cuticular water loss so that the contribution of the urnulae secretion can be assessed. Because killed mites are used (eliminating respiratory artifacts) for correct critical transition temperature determination (Wharton, 1985; Hadley, 1994), the water loss that is measured is solely cuticular. The observation that the activation energy (E_a) is lower for mites that secreted than mites that have not secreted indicates that if the secretion is present on the cuticular surface, then the amount of water loss occurring from one test temperature jump to the next is proportionately less when the secretion is present. Without the secretion present, no extra water-proofing is available to decrease the amount of water loss from one temperature jump to the next, resulting in a steeper slope and consequently a higher activation energy. The activation energy for water loss is not an activation energy in the truest sense of the word because it does not reflect a process that is limiting, nor does it respond to an artificial rate increase. However, it is more appropriately described as a permeability constant (Yoder *et al.*, 2005a). Permeability constants are best described as lower activation energies for water loss that correlate with lower rates of water loss and generally coincide with cuticular modifications that are suited for enhancing water conservation (Yoder and Houck, 2001; Benoit *et al.*, 2005, 2008). Thus, the lower activation energy for mites that have secreted indicates that the secretion is reducing the proportionate amount of water loss that occurs as the temperature rises, and denotes that it represents a major cuticular modification. Not only does the secretion cause a reduction in the amount of water loss progressively from one temperature to the next, but there is a reduction in the amount of water loss overall. This overall reduction in water loss due to the presence of the secretion is reflected by the lowering on the y -intercept of where the regression line crosses on the Arrhenius plot which is de-

fined as frequency (steric) factor A . The lowering in steric factor A is simply an integrated measure of decreased water loss as a result of the presence of the secretion, perhaps in relation to greater viscosity of the urnulae secretion; thus, overall integumental water loss is reduced. In living mites, this overall reduction is associated with lower net transpiration rates (integumental plus respiratory water loss) for mites that have secreted because the integumental component has been suppressed that enhances water conservation.

There is no question that adult female *Balaustium* sp. mites experience rapid desiccation once temperature exceeds 50-52 °C, and although this is determined in killed mites so that the focus is on the cuticle, this temperature threshold where evaporative water losses accelerate sharply has biological value because it correlates with a marked decrease in survival, known as the lethal permeability temperature, LPT (Yoder *et al.*, 2007a). The curvature in the linear plot is linked to the temperature where the mites succumb to heat stress (Yoder *et al.*, 2007a). As a result of urnulae secretion, not only is the overall amount of passive water loss suppressed (the entire biphasic relation is shifted down the ordinate), but the abrupt water loss that is potentially lethal does not occur until temperatures reach or exceed 58-60 °C. Thus, urnulae secretion cuts down on evaporative losses until higher temperatures are reached; thus survivorship is considerably enhanced at higher temperatures. Therefore, mites are less liable to die by temperature-induced dehydration and water stress because of the presence of the urnulae secretion. It is important to note that the 50-52°C (mites that did not secrete) and 58-60 °C (mites having secreted) are not critical transition temperatures (CTT) because the kink depicting activation energy (upon which CTT is based) is not present on the Arrhenius plot. The presence of urnulae secretion helps to minimize evaporative losses thereby enhancing the mites ability to function properly longer when exposed to high temperature and safeguards against large, lethal evaporative losses affording greater resistance to heat stress by causing an upward shift in the lethal permeability temperature (LPT, corresponding to the maximum temperature permitting survival) and simultaneously decreasing total body water losses.

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