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RESEARCH ARTICLE

Effect of cuticular abrasion and recovery on water loss rates in queens of the desert harvester ant *Messor pergandei*

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SUMMARY

Factors that affect water loss rates (WLRs) are poorly known for organisms in natural habitats. Seed-harvester ant queens provide an ideal system for examining such factors because WLRs for mated queens excavated from their incipient nests are twofold to threefold higher than those of alate queens. Indirect data suggest that this increase results from soil particles abrading the cuticle during nest excavation. This study provides direct support for the cuticle abrasion hypothesis by measuring total mass-specific WLRs, cuticular abrasion, cuticular transpiration, respiratory water loss and metabolic rate for queens of the ant *Messor pergandei* at three stages: unmated alate queens, newly mated dealate queens (undug foundresses) and mated queens excavated from their incipient nest (dug foundresses); in addition we examined these processes in artificially abraded alate queens. Alate queens had low WLRs and low levels of cuticle abrasion, whereas dug foundresses had high WLRs and high levels of cuticle abrasion. Total WLR and cuticular transpiration were lowest for alate queens, intermediate for undug foundresses and highest for dug foundresses. Respiratory water loss contributed ~10% of the total WLR and was lower for alate queens and undug foundresses than for dug foundresses. Metabolic rate did not vary across stages. Total WLR and cuticular transpiration of artificially abraded alate queens increased, whereas respiratory water loss and metabolic rate were unaffected. Overall, increased cuticular transpiration accounted for essentially all the increased total water loss in undug and dug foundresses and artificially abraded queens. Artificially abraded queens and dug foundresses showed partial recovery after 14 days.

Key words: ant queen, artificial abrasion, CO_2 release rate, cuticle abrasion, cuticle repair, cuticular transpiration, mating stage, metabolic rate, respiratory water loss.

INTRODUCTION

Water availability is one of the most important factors affecting the distribution of insects because their small size and high surface to volume ratio make them prone to desiccation. Consequently, finding insects in virtually all terrestrial habitats is a testament to their numerous adaptations to maintain water balance. One of the most important adaptations is a waterproof layer of hydrophobic lipids that cover the cuticle, which is typically the primary avenue of water loss (Chown and Nicolson, 2004; Hadley, 1994). The abiotic environment exerts strong selective pressure on the amount and type of these hydrophobic lipids, as shown by comparative studies demonstrating that water loss rates (WLRs) are often positively associated with increasingly mesic conditions in insects (e.g. Gibbs et al., 2003; Gibbs and Matzkin, 2001; Hadley and Schultz, 1987; Hood and Tschinkel, 1990; Klok and Chown, 2003; Massion, 1983; Parkash et al., 2008; Studier and Lavoie, 1990) and other arthropods (Hadley et al., 1981; Toolson and Hadley, 1977; Worland and Block, 1986). The positive association between WLR and environmental humidity can also vary through the life cycle, e.g. occurring in pupae but not in adults, indicating that selection exerts more pressure on some life stages than on others (Kleynhans and Terblanche, 2008). However, the association between water loss and environment is not universal because other factors, such as phylogenetic constraints, also exert a strong effect on water balance and WLRs (Grefen and Ar, 2004; Grefen and Ar, 2006). The correlation between water loss and environment is also inconsistent at the intra-specific level because cuticular lipids correlate with desiccation resistance across populations in some species (Foley and Telonis-Scott, 2011) but not in others (Jurenka et al., 2007).

Despite the numerous studies on mechanisms and patterns of water loss, we still have a poor understanding of factors that affect water balance or cause variation in WLRs for organisms in natural habitats (see Chown and Nicolson, 2004; Edney, 1977). However, at least four factors are known to affect water balance for organisms in the field: (1) activity, (2) grouping, (3) temperature and (4) abrasion. Activity increases metabolic rate and respiratory water loss, but it should not be associated with cuticular transpiration (Bursell, 1959; Machin et al., 1991). Grouping decreases water loss by decreasing surface area and/or increasing local humidity, and the advantages of grouping generally increase with group size (Klok and Chown, 1999; Rasa, 1997; Yoder and Grojean, 1997; Yoder and Smith, 1997). Temperature also has a dramatic direct effect on cuticular transpiration (Hadley, 1994) and can also increase the potential for water stress by melting cuticular lipids at temperatures that are ecologically relevant (Gibbs, 2002). Lastly, soil-dwelling insects can experience cuticle abrasion as a result of excavating or traversing through tunnels (Johnson, 2000c; Johnson and Gibbs, 2004). Several experimental studies using inert dusts demonstrated

that artificial abrasion increases WLRs (Collins, 1969; Hafez et al., 1970; Holdgate and Seal, 1956; Nel, 1965; Wigglesworth, 1945), but none of these studies linked their laboratory results with abrasion for field-collected insects. Additionally, few data exist on the ability of insects to repair damage caused by abrasion or the time course of such repair (Hafez et al., 1970; Wigglesworth, 1945; Wolfe, 1955), and none of these studies examined field-collected organisms to control for changes in respiratory water loss.

Ant queens provide an ideal system to examine many aspects of water balance for field-collected organisms because WLRs vary by mating stage: WLRs are lowest for unmated, alate queens and increase twofold to threefold for mated, dealate queens excavated from their incipient nest (dug foundresses) (Johnson, 2000c; Johnson and Gibbs, 2004). Determining the mechanisms that cause increased WLRs for dug foundresses is a first step toward understanding water balance dynamics in natural habitats during the most crucial and vulnerable stage in the life of an ant colony (Hölldobler and Wilson, 1990; Johnson, 1998). Indirect data suggest that increased WLRs in dug foundresses result from soil particles scratching and/or displacing cuticular hydrocarbons during the process of nest excavation, causing furrows that enhance transpiration (Johnson, 2000c; Johnson and Gibbs, 2004). It is unknown whether higher WLRs for dug foundresses are permanent or if they can be ameliorated by repair.

The present study used queens of *Messor pergandei* Mayr to directly test the hypothesis that nest excavation abrades the cuticle and thereby causes dramatically increased WLRs, whereas discontinuing excavation allows cuticular repair and a decrease in WLRs. This species is especially well suited for such study because desiccation causes mortality for dug foundresses (R.A.J., unpublished data). Three predictions of this hypothesis are: (1) dug foundresses will have higher total WLRs and a higher cuticular transpiration than other mating stages, (2) the cuticle of dug foundresses will show more damage than other mating stages and (3) dug foundresses will have a reduced cuticular transpiration after a recovery period without digging. We tested these predictions by quantifying cuticular damage using scanning electron micrographs and measuring WLRs from alate queens and dug foundresses. To control for other contributors to water loss, we measured metabolic rate, activity, cuticular transpiration and respiratory water loss by flow-through respirometry, and total WLRs were measured gravimetrically. We first assessed recovery by dug foundresses, which were abraded naturally while digging their nests. We then used a two-way experiment (three treatment groups across three time intervals) to measure the effect of artificial abrasion on alate queens and their ability to recover from such abrasion.

MATERIALS AND METHODS

Study organism

Messor pergandei (subfamily Myrmicinae) is a soil-nesting ant that inhabits the Sonoran and Mohave Deserts, typically in sandy soils at elevations lower than 900 m – areas that collectively encompass the hottest, most arid portions of North America (Creighton, 1953; Johnson, 1992; Johnson, 2000b). The colony cycle begins with the mating flights, which occur from mid-January to mid-March (Cahan, 2001; Pollock and Rissing, 1985). Mating occurs in the air; the mated queen then tears off her wings and searches for a site to excavate her new nest. She then remains in her nest, and metabolizes body reserves to support herself and rear her first brood of workers, which emerge after ~4 weeks at 30°C (R.A.J., unpublished data). At our collection site, unrelated foundresses cooperate during nest founding, whereas in other areas single queens initiate nests (Johnson, 2000a).

Collection of study animals

We collected *M. pergandei* queens at three mating stages: (1) unmated alate queens were collected from their natal nest (alates); (2) mated, dealate queens were collected on the ground immediately after mating, but before beginning to excavate their nests (undug foundresses); and (3) mated, dealate queens were excavated from their incipient nest several days after the mating flight (dug foundresses). Queens were collected from mid-February to late-March from ~0.5 km east of the intersection of Interstate-10 and McCartney Road, Pinal County, AZ (32° 56′N, 111° 42′W; elevation 440 m), USA. Individuals were placed in closed containers with moist paper towels to maintain hydration and transported to the laboratory. All animal experiments were performed according to the relevant USA laws.

WLRs and cuticle damage for alate queens and dug foundresses

We determined mass-specific WLRs of alate queens and dug foundresses within 48 h of capture. These data reflect natural WLRs because individuals did not have time to acclimate to laboratory conditions (Lighton and Feener, 1989; Spicer and Gaston, 1999). Mass-specific WLRs were determined by enclosing each ant in a 20×7 mm chamber made of rigid plastic tubing sealed on both ends with push-fit caps of stainless steel screen. Each chamber was weighed with caps to 0.01 mg on an analytical microbalance (AND GR-202, A&D, San Jose, CA, USA). One cap was opened, an ant was picked up by a wing (alates) or by a leg (dealates) using forceps and inserted into the chamber, and the chamber was sealed and reweighed. Columns were assembled by connecting the chambers in series using short lengths of flexible plastic tubing; each column contained up to 15 chambers plus one empty control chamber. Columns were then placed in a darkened constant-temperature cabinet at 30±0.5°C. Air, desiccated using Drierite (Drierite, Xenia, OH, USA), was forced through the columns at a rate of 100–150 ml min^{-1}, controlled by a needle valve and rotameter (Gilmont Instruments, Barrington, IL, USA). Chambers were weighed after 6–8 h, depending on the experiment. Final mass of each ant was adjusted by the mean change in mass of control chambers (Johnson, 2000c). We assumed that mass loss over each trial was equivalent to water loss (Duncan and Lighton, 1994; Edney, 1977). After water loss trials, ants were frozen at –75°C so that they could be used to quantify cuticular damage (see below).

Mass-specific WLRs (μg H_2O mg^{-1} wet mass h^{-1}) were calculated for each individual. Mass-specific WLRs were then compared between alate queens and dug foundresses using a *t*-test.

We quantified cuticular damage on these same queens using micrographs taken with a field emission scanning electron microscope (SEM; Leica Stereoscan 360FE, Solms, Germany) set at 2.5 kV. Each queen was removed from the ultracold freezer, attached to an aluminum specimen mount with colloidal graphite, and then placed in the SEM chamber. Data were standardized across queens by taking micrographs along the medial dorsal surface at three locations: (1) the clypeus, (2) immediately anterior to the median ocellus and (3) the pronotum immediately posterior to the suture with the pronotal collar (Fig. 1). Micrographs were taken at 250× magnification and encompassed an area of 0.248 mm^2.

Micrographs were taken for randomly chosen alate queens and dug foundresses (N=10 for each mating stage) with the caveat that mass-specific WLRs (μg H_2O mg^{-1} h^{-1}) were <8.5 for alate queens (20 of 42 individuals tested) and >14.0 for dug foundresses (45 of 53 individuals tested). This restriction enhanced our ability to detect differences in cuticular damage between the two mating stages. We

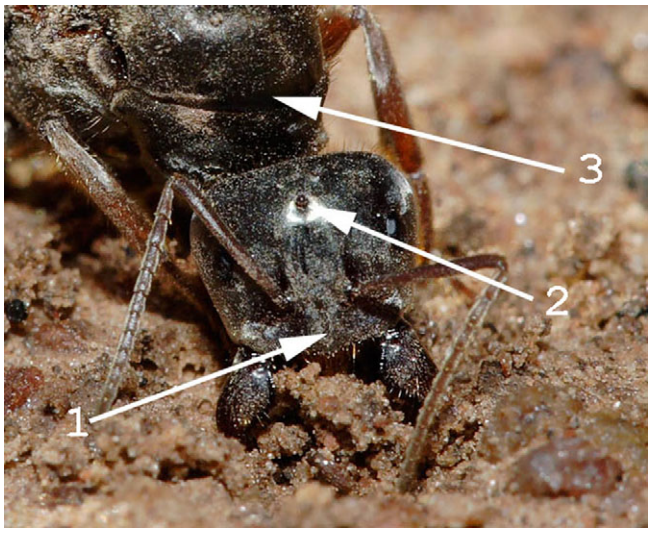

Fig. 1. Head and thorax of a queen seed-harvester ant, *Messor pergandei,* showing the locations at which we quantified cuticular abrasion using scanning electron micrographs: 1, the clypeus; 2, immediately anterior to the median ocellus; and 3, the pronotum immediately posterior to the suture with the pronotal collar. Artificial abrasion involved lightly rubbing each queen with emery paper 10 times on the head and anterior dorsum of the mesosoma.

used CorelDraw 8.0 to place a 13×14 grid (182 cells) on each micrograph, and two authors (R.A.J. and A.K.) independently counted damaged sites across all cells for all queens. Counts of cuticular damage were compared between alate queens and dug foundresses at each of the three sites using a *t*-test followed by a Bonferroni sequential correction.

Effect of mating stage and vital state on WLR

Mating and colony founding by queens of *M. pergandei* involve a series of activities that include flying from the nest, mating in the air (with *in copulo* pairs sometimes falling to the ground), tearing off their wings, then excavating a nest and laying eggs (R.A.J., personal observation). Vital state (live *versus* dead) is also an important component for understanding insect water balance because an unknown active control mechanism often results in lower WLRs for live than for dead individuals (Hadley, 1994). Such active control occurs in alate queens, but not in dug foundresses of *M. pergandei* (Johnson, 2000c), but has not yet been investigated for undug foundresses. Consequently, we determined mass-specific gravimetric WLRs for live and dead [hydrogen cyanide (HCN)-killed] alate queens, undug foundresses and dug foundresses.

For alate queens, we collected 30 individuals from one colony, and then randomly divided them into live and HCN-killed treatment groups; this method controlled for colony variation and test conditions. Few undug foundresses were available (N=18), so we measured total mass-specific WLRs on live individuals, and 24 h later we used the same individuals in the HCN-killed treatment. To minimize loss of liquids prior to HCN exposure, individuals were cooled to immobility, asphyxiated with CO_2, and then exposed to HCN. The oral cavity was then sealed with paraffin, and WLRs were determined gravimetrically (see above). We also measured mass-specific WLRs of live and HCN-killed alate queens that had been artificially abraded, which provided experimental data that could be compared with that of dug foundresses. Artificial abrasion involved lightly rubbing each queen with emery paper (280 mesh, Norton Brand, Worcester, MA, USA) 10 times on the head and anterior dorsum of the mesosoma (see Fig. 1).

Variation in mass-specific WLR (dependent variable) across mating stages and vital states (independent variables) was analyzed using a two-way ANOVA; we excluded artificially abraded alate queens from this analysis so that we could determine the pattern of variation for untreated queens. A one-way ANOVA was then used to determine differences among treatment groups; the one-way ANOVA was run with and without artificially abraded alate queens.

Flow-through respirometry

We used a flow-through respirometry system to measure WLR and metabolic rate (as CO_2 release rate). Our system used compressed air, which was forced through a Drierite, soda lime, Drierite column to remove CO_2 and water vapor, and then through the respirometry system at a rate of 200 ml min^{-1}, as controlled by mass flow controllers (Tylan 2800, Tylan General Inc., San Diego, CA, USA). The amount of water and soda lime in the artificially produced medical gas (Matheson Tri-Gas, Phoenix, AZ, USA) was minimal (0–0.5 p.p.m. CO_2; 0–0.1 p.p.t. water vapor). The combination of chemicals in the scrubber column was sufficient to remove any potential remnants. Additionally, the soda lime produces a small amount of water when it reacts with CO_2, thus driving the reaction for CO_2 removal. The air was flushed through the reference chamber of a differential CO_2 and H_2O infrared absorption analyzer (LI-7000, Licor Systems, Lincoln, NE, USA). The air then entered an eight-channel multiplexer [Sable Systems International (SSI), Las Vegas, NV, USA), which provided one of seven glass respirometry chambers (50×12 mm) with the controlled air stream. One chamber was omitted to reduce the cycle time for the analyzed animals. While one chamber was analyzed, the other six chambers were flushed with dry, CO_2-free air at a rate of 20–50 ml min^{-1} to avoid hypoxia or accumulation of CO_2 between runs. Each ant was weighed to 0.01 mg, and then placed into one of the six chambers; one control chamber remained empty to obtain a baseline reading for later calibration. Chambers were then placed in a temperature-controlled cabinet (PTC-1, SSI) at 30±0.1°C. The entire system was fitted with Bev-A-Line tubing to reduce absorption of CO_2 and water vapor. Data were collected with ExpeData Software (SSI) after an analog to digital signal transformation (UI-2 interface; SSI). The respirometer was calibrated to zero levels with high purity medical grade gas (0.1 p.p.m. CO_2 and 0.5 p.p.m. water vapor; Matheson Tri-Gas), scrubbed from remnant water vapor with a magnesium perchlorate column. The CO_2 channel was calibrated to 100 p.p.m. CO_2 and the water channel was calibrated to 8.053 p.p.t. with a humidifier–water trap combination set to 30 and 10.8°C, respectively.

Chambers containing the ants were placed in the respirometry system for an acclimation interval of 30 min. An automated time sequence in ExpeData then switched the multiplexer channels through the seven respirometry chambers. One multiplexing sequence consisted of an initial 20 min reading of the control chamber, a 30 min reading of each animal and a 10 min reading of the control chamber, for a total of 3.5 h. This sequence was repeated at least three times per queen. Values for CO_2 and water vapor were corrected by the control chamber value, and the sequences for each queen were pooled. Ants were reweighed after each run; estimates of water loss during the run, as calculated from mass loss, were consistent with water loss rates determined from water-vapor analysis in flow-through measurements. The lag between switching the multiplexer and reading the bulk of air arriving at the analyzer was ~5 s. CO_2 readings stabilized after several seconds and the water

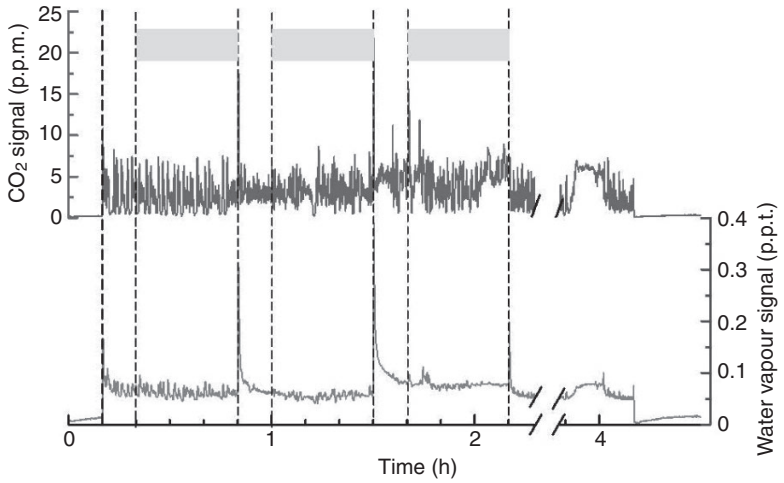

Fig. 2. Raw recording trace of CO_2 release and water vapor loss for alate queens. Note the break in the *x*-axis; only data from three of the six recorded chambers are presented in full. One chamber was recorded for 40 min; the first 10 min of the recording for each chamber were omitted from analysis because this was a period of equilibration of the flow through respirometry setup. The remaining 30 min time-block (gray bars) was used for data analysis.

vapor reading was stable after 5–7 min. Thus, the first 10 min of every 40-min reading were omitted from analysis, resulting in 30 min of analyzable data per animal per cycle (Fig. 2).

A primary goal of this study was to assess the relative contribution of cuticular transpiration and respiratory water loss. This goal was facilitated by our ability to partition cuticular transpiration and respiratory water loss using a statistical technique that involved regressing WLR against CO_2 release for each ant and extrapolating the regression line down to the point of spiracular closure (see Gibbs and Johnson, 2004). The regression used 5 s time-averaged, lag-corrected values over the 30 min respirometry trials. Water loss rate (mg h^{-1}) at the intercept, where CO_2 release (μl h^{-1}) equals zero, was assumed to represent cuticular transpiration. Respiratory water loss was calculated as the difference between total water loss and cuticular transpiration (see Gibbs and Johnson, 2004). Occasionally, queens were highly active and displayed continuous rather than cyclic or discontinuous CO_2 release; in these sequences, the regression typically resulted in low correlation coefficients and negative slopes. These sequences were omitted from analyses.

Effects of activity on WLR

In all respirometry trials, activity of each queen was recorded by an in-house-constructed infrared LED light barrier with the LED and photosensor mounted in the front and back lid of each chamber, respectively. Activity of each animal was determined by calculating the absolute difference sum (ADS) of the activity data (Lighton and Turner, 2004), and these data were then compared across treatments using a paired-sample *t*-test, one-way ANOVA or repeated-measures ANOVA.

Effect of mating stage and recovery on WLR for field-collected queens

We assessed the effect of mating stage using a second experiment in which we measured WLRs gravimetrically on an additional set of alate queens, undug foundresses and dug foundresses (see above). Each individual was then placed in a water tube (a test tube that was partially filled with water trapped by a cotton plug, with a second cotton plug placed over the opening of the tube), which allowed *ad libitum* access to water. After 24 h, we selected a subset of queens from each mating stage to determine mass-specific WLR, metabolic rate and activity using flow-through respirometry. The only caveat was that alate queens had a mass-specific WLR <8.5 (μg H_2O mg^{-1} h^{-1}) and dug foundresses had a mass-specific WLR >14.0 (see above). Variation in mass-specific WLR, cuticular transpiration, respiratory water loss and CO_2 release (dependent variables) was then compared across mating stages (independent variable) using a separate one-way ANOVA for each variable.

We also assessed the ability of dug foundresses to recover from cuticular damage caused by excavating a nest. We measured mass-specific WLRs gravimetrically within 48 h of excavation, placed each queen in a water tube to rehydrate for 24 h, and then measured WLR, CO_2 release rate and activity for a subset of these foundresses using flow-through respirometry (see above). Each foundress was then placed in a water tube and maintained in a darkened incubator at 30°C. After 14 days, we remeasured mass-specific WLR gravimetrically; foundresses previously measured using flow-through respirometry were remeasured 24–48 h later. Trials lasted 6 h for both gravimetric measures in order to reduce desiccation mortality. Total mass-specific WLRs, cuticular transpiration, respiratory water loss rate, metabolic rate, and activity were then compared between the post-excavation period and the 14-day recovery period using a paired-sample *t*-test.

Effect of artificial abrasion and recovery on WLR for alate queens

We examined the effect of abrasion and recovery by measuring total mass-specific WLRs for alate queens across three time intervals (pre-abrasion, post-abrasion and 14-day recovery). We first used our gravimetric setup to screen an additional set of queens; only those that had a mass-specific WLR <8.5 (μg H_2O mg^{-1} h^{-1}) were used. This restriction standardized our experiment so that we included only undamaged alate queens that had similar mass-specific WLRs, thus allowing us to better control pretreatment WLRs across the three treatment groups. After 24 h, we used flow-through respirometry to measure mass-specific WLRs, metabolic rate and activity on a subset of these queens. After collecting pre-abrasion data, each queen was randomly treated in one of three ways: (1) no treatment, (2) lightly rubbed with Whatman filter paper 10 times on the head and anterior dorsum of the mesosoma and (3) lightly rubbed with emery paper 10 times on the head and anterior dorsum of the mesosoma (see Fig. 1). We re-measured mass-specific WLR gravimetrically for each individual ~24 h after treatment. Trials lasted 6 h to reduce desiccation mortality; queens previously measured using flow-through respirometry were re-measured 24–48 h later. We examined recovery from abrasion by measuring mass-specific WLRs gravimetrically after 14 days; queens previously measured using flow-through respirometry were re-measured 24–48 h later.

During inter-trial periods, each queen was placed in a water tube to allow rehydration and maintained in a darkened incubator at 30°C. Only queens that survived throughout the experiment were included in analyses.

Data were analyzed across the three times (pre-abrasion, post-abrasion, 14-day recovery) using a one-way repeated-measures ANOVA (SPSS, 1990); time was the within-subjects effect and treatment (no treatment, filter paper, emery paper) was the between-subjects effect. Each of the four dependent variables (mass-specific WLR, cuticular transpiration, respiratory water loss, metabolic rate) was analyzed using a separate repeated-measures ANOVA. An *a posteriori* one-way ANOVA followed by a Duncan's multiple range test determined the nature of within- and between-subjects differences. For all statistical tests, data were transformed as necessary, to meet the assumptions of normal distribution and homogeneity of variance.

RESULTS

WLR and cuticle damage for alate queens and dug foundresses

In agreement with prediction 1 of our hypothesis, mass-specific WLRs ($\mu g\,H_2O\,mg^{-1}\,h^{-1}$) were significantly higher for dug foundresses (21.4±0.7; mean ± s.e.m.) than for alate queens (9.8±0.4; *t*-test, t_{93}=15.4, P<0.001; data log transformed). This is in agreement with the pattern documented by Johnson (Johnson, 2000c). In a similar manner, mass-specific WLRs were significantly higher for dug foundresses (29.2±1.4) than for alate queens (7.4±0.3; t_{18}=23.3, P<0.001; data ln transformed) that were used to quantify cuticular damage. In agreement with prediction 2 of our hypothesis, when the micrographs were examined both authors (R.A.J. and A.K.) counted a significantly higher number of damaged sites on dug foundresses than on alate queens at all three locations on the cuticles (*t*-test, t_{18}>8.6 for all comparisons, P<<0.001; Figs 3, 4), even after correcting *P*-values using a Bonferroni sequential correction.

Effect of mating stage and vital state on WLR

Mass-specific WLRs increased across progressive mating stages, and WLRs were also higher for dead than for live queens. However, the values for vital state showed a different pattern across the three mating stages, resulting in a significant interaction between mating stage and vital state (Table 1). A one-way ANOVA across all treatment groups following a reciprocal transformation of the data demonstrated that mass-specific WLR was lowest for live alate queens, intermediate for HCN-killed alate queens and live and HCN-killed undug foundresses, and highest for live and HCN-killed dug foundresses (Fig. 5). The second one-way ANOVA showed that total mass-specific WLR was significantly higher for live and HCN-killed artificially abraded alate queens than for all other treatment groups (Fig. 5). Queen wet mass in this experiment was 39.4±0.3 mg for alate queens, 35.8±0.6 mg for both undug and dug foundresses, and 37.1±0.4 mg for artificially abraded alate queens.

Effect of mating stage and recovery on WLRs for field-collected queens

In the second experiment, mass-specific WLR varied across the three mating stages, but the patterns differed for gravimetric measures and respirometry data. For gravimetric data (Fig. 6A; one-way ANOVA, $F_{2,499}$=1931.3, P<0.001), mass-specific WLRs were lowest for undug foundresses, intermediate for alate queens and highest for dug foundresses (Duncan's multiple range test, P<0.001). Mass-specific total WLRs calculated from respirometry data also varied across the three stages ($F_{2,36}$=48.8, P<0.001), and they were lowest for alate queens, intermediate for undug foundresses and highest for dug foundresses (Duncan's range test, P<0.05). Respirometry data also demonstrated that cuticular transpiration and respiratory water loss varied across the three mating stages (cuticular transpiration: $F_{2,36}$=47.0, P<0.001; respiratory water loss: $F_{2,36}$=5.6, P=0.007). Cuticular transpiration was lowest for alate queens, intermediate for undug foundresses and highest for dug foundresses (Fig. 6B; P<0.05), whereas respiratory water loss was lowest for alate queens and undug foundresses and significantly higher for dug foundresses (Fig. 6C; P<0.05). One outlier for dug foundresses contributed heavily to this ANOVA result for respiratory water loss: removal of this outlier increased the *P*-value from 0.007 to 0.09. Metabolic rate and activity level did not vary across the three mating stages (metabolic rate: $F_{2,36}$=0.04, P=0.96; Fig. 6D; activity level: $F_{2,36}$=1.41, P>0.25). Queen mass varied in a predictable manner

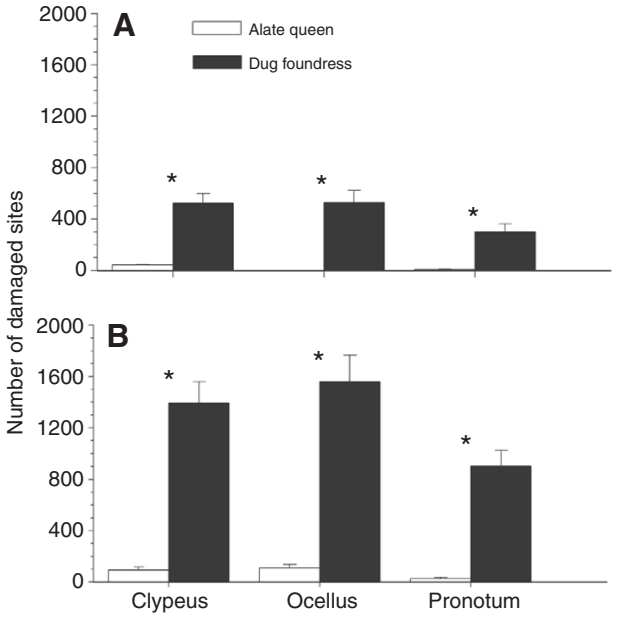

Fig. 3. Quantification of cuticular damage at three locations on alate queens and dug foundresses (see text for details); counts were made by (A) R.A.J. and (B) A.K. Values are means ± 1 s.e.m. (N=10 per group). Asterisks denote significant differences (P<<0.0001) based on a *t*-test followed by a Bonferroni sequential correction. Micrographs of representative queens are shown in Fig. 4.

Table 1. Results of a two-way ANOVA for the effect of mating stage and vital state (independent variables) on mass-specific water loss rates ($\mu g\,H_2O\,mg^{-1}\,h^{-1}$; dependent variable) by queens of the seed-harvester ant *Messor pergandei*

		Water loss rate		
Source of variation	d.f.	MS	*F*	*P*
Corrected model	5	5504.4	17.92	<0.001
Mating stage	2	11699.3	38.09	<0.001
Vital state	1	3225.8	10.50	0.002
Mating stage × vital state	2	964.0	3.14	0.049
Error	80			
Total	86			
R^2			0.53	

MS, mean square.
N=13–16 per treatment. See also Fig. 5.

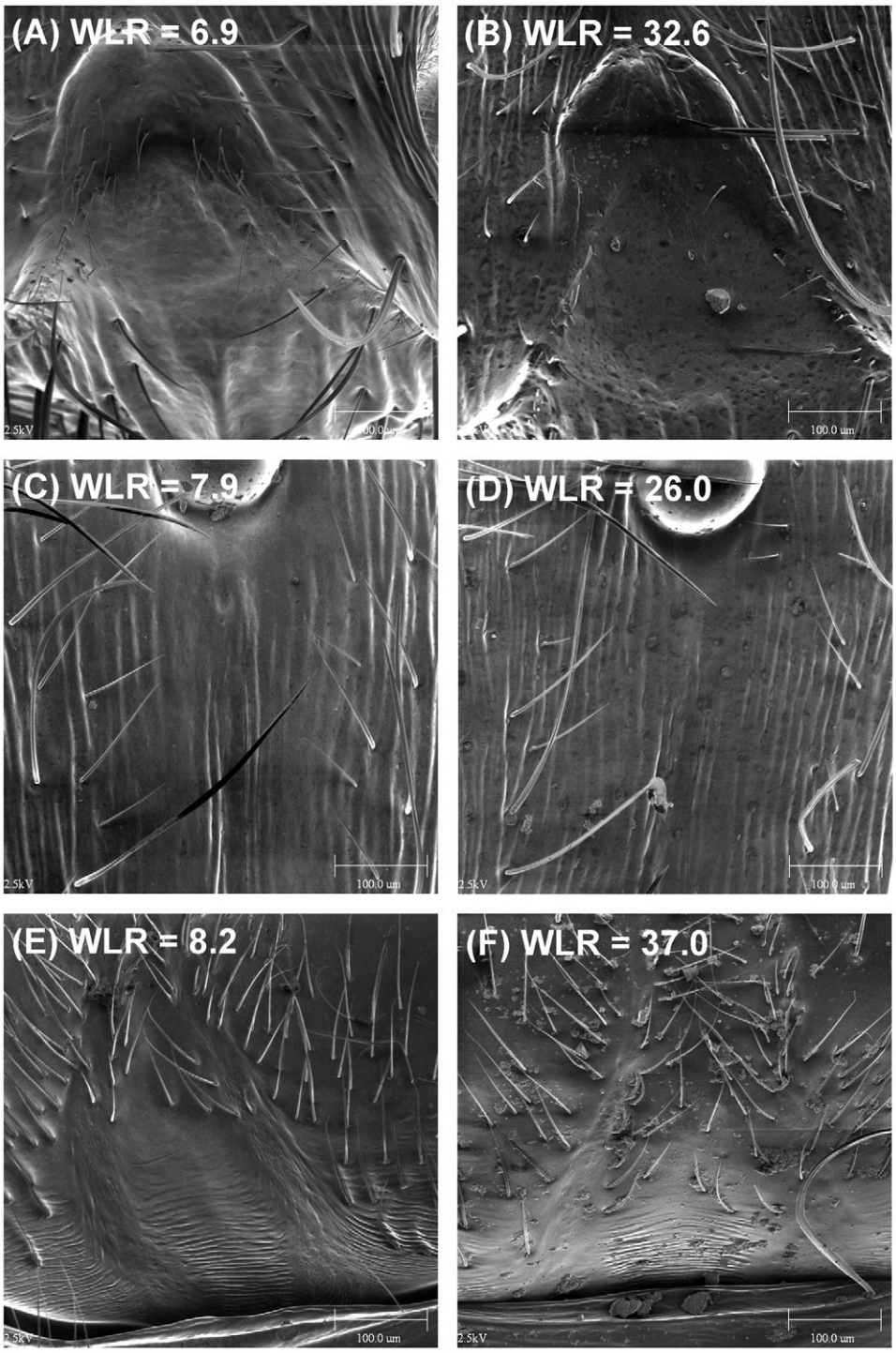

Fig. 4. Representative micrographs of (A,B) the clypeus, (C,D) the area immediately anterior to the median ocellus and (E–F) the pronotum immediately posterior to the suture with the pronotal collar of (A,C,E) alate queens (undamaged) and (B,D,F) dug foundresses (damaged; see text for details). The mass-specific water loss rate (WLR; $\mu g\,H_2O\,mg^{-1}\,h^{-1}$) is given at the top left of each panel.

over the three mating stages, decreasing from 39.3±0.2 mg for alate queens, to 37.4±0.4 mg for undug foundresses, to 36.2±0.02 mg for dug foundresses.

After 14 days recovery, dug foundresses displayed a significant decrease in total mass-specific WLR (gravimetric data: paired-sample *t*-test, t_{17}=5.75, P<0.001; respirometry data: t_5=12.8, P<0.001; Fig. 7A). Respirometry data indicated that cuticular transpiration and metabolic rate decreased significantly over the 14-day interval (cuticular transpiration: paired-sample *t*-test, t_5=10.0, P<0.001; Fig. 7B; metabolic rate: t_5=3.75, P=0.013; Fig. 7D). Respiratory water loss remained similar over this interval (t_5=1.43, P=0.21; Fig. 7C). Activity level did not differ between the two time intervals (paired-sample *t*-test, t_5=1.11, P>0.30).

The lower metabolic rate after recovery suggests that the queens acclimated to laboratory conditions over the 14-day interval. Consequently, we assessed the effect of acclimation on respiratory water loss using a regression model that included foundress stage as the independent variable, respiratory water loss as the dependent variable, and metabolic rate as a covariate. Foundress stage was not associated with respiratory water loss ($F_{1,9}$=1.53, P>0.24), but metabolic rate was significantly associated with respiratory water loss ($F_{1,9}$=8.59, P<0.02). These results indicate that respiratory water loss

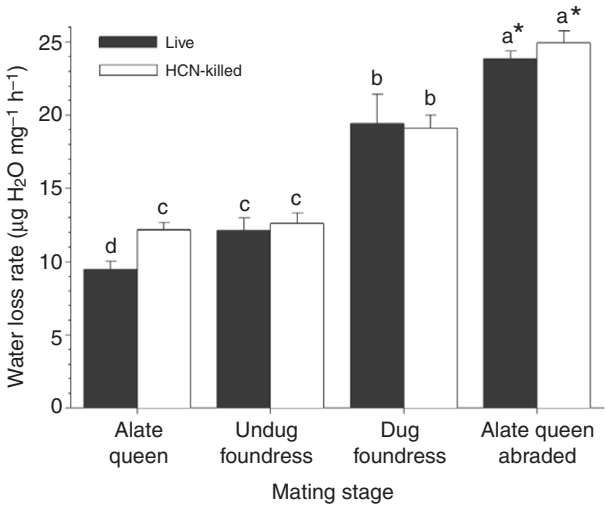

Fig. 5. Mass-specific water loss rate (μg H_2O mg^{-1} h^{-1}) at 30°C for live and HCN-killed queens. Significant differences among treatment groups (P<0.05) are denoted by the letters *a–d*: *a>b>c>d*. Groupings are based on a one-way ANOVA followed by a Duncan's multiple range test (see text for details). N=13–16 per treatment. Mating stages: alate queen, queens collected from their natal nest; undug foundress, queens captured on the ground after mating but before beginning nest excavation; dug foundress, queens collected from incipient nests several days after beginning nest excavation; alate queen abraded, alate queens that were artificially abraded with emery paper. *These two groups were excluded from the initial one-way ANOVA (see text).

rate did not vary over the 14-day recovery interval even when accounting for differences in metabolic rate. Wet mass of dug foundresses decreased significantly over the 14-day period, by an average of 2.3% of the initial mass ($mass_{day\ 1}$=36.22±0.07 mg; $mass_{day\ 14}$=35.39±0.08 mg, t_{17}=2.77, P=0.013). The lower mass on day 14 did not contribute to the observed decrease in total mass-specific WLR, but rather we would have expected a larger difference between WLRs of the two groups if body mass had remained constant.

Effect of artificial abrasion and recovery on WLRs for alate queens

A repeated-measures ANOVA revealed that artificial abrasion resulted in significant within- and between-subjects effects for total WLRs. The between-subjects effect indicated that WLRs were significantly higher for the emery paper treatment than for the two other treatments, whereas the within-subjects effect indicated that WLRs were significantly higher for the post-abrasion measurement than for the pre-abrasion or 14-day recovery measurements. Consequently, there was also a significant within-subjects interaction effect between treatment and time (Table 2). An *a posteriori* one-way ANOVA ($F_{8,168}$=8.92, P<0.001), followed by a Duncan's multiple range test demonstrated that abrasion with emery paper significantly increased total mass-specific WLRs (P<0.05), and that mass-specific WLRs for this same treatment cell decreased significantly (~30.0%) after the 14-day recovery period (P<0.05), although it remained significantly higher than control and filter paper treatments (Fig. 8A). Activity level did not vary in the artificial abrasion experiment (repeated-measures ANOVA, $F_{6,32}$=1.78, P>0.13). Queen wet mass in the respirometry experiment was 30.4±0.6 mg for the pre-abrasion treatment, 29.4±0.6 mg for the post-abrasion treatment and 27.2±0.7 mg after the 14-day recovery interval.

For cuticular transpiration, treatment (between-subjects effect) was not significant, whereas the within-subjects effect indicated that cuticular transpiration was significantly higher for the post-abrasion measurement than for the pre-abrasion or 14-day recovery measurements. This resulted in a significant interaction between time

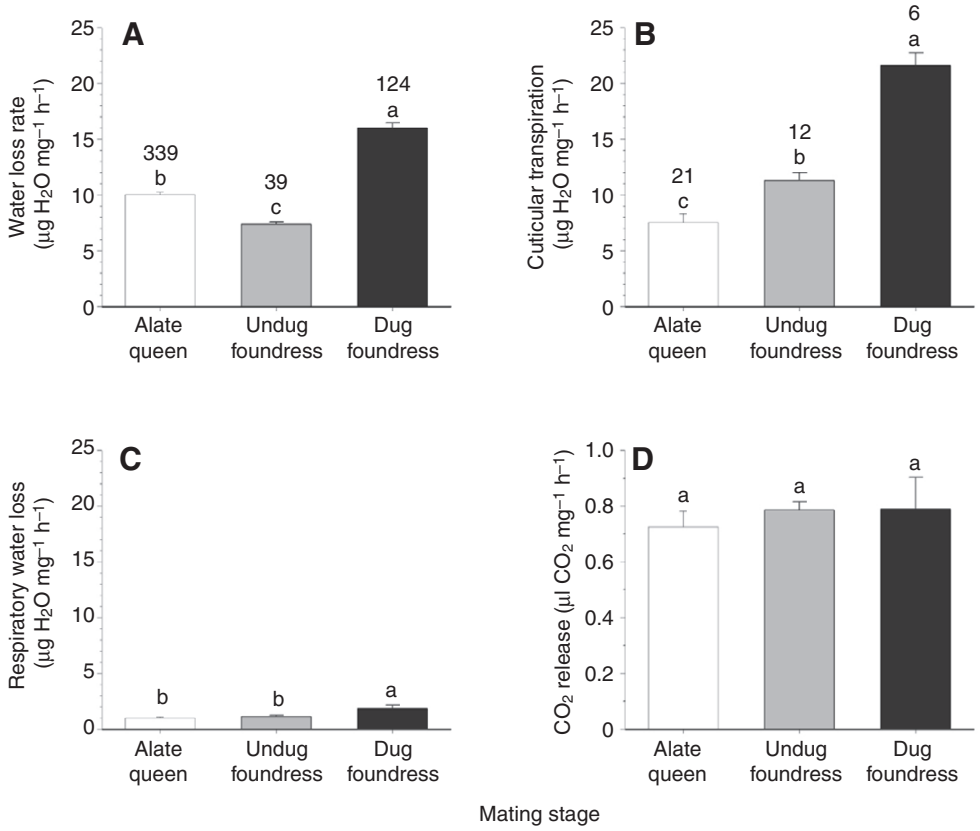

Fig. 6. Effect of mating stage on (A) total mass-specific water loss rate (WLR), (B) cuticular transpiration, (C) respiratory water loss (μg H_2O mg^{-1} h^{-1}) and (D) carbon dioxide release (μl CO_2 mg^{-1} h^{-1}) of queens. Significant differences within each panel are denoted by the letters *a–c*: *a>b>c*. Groupings are based on a one-way ANOVA followed by a Duncan's multiple range test. Sample size is given above each stage; sample size is the same in B–D. For comparison, total mass-specific WLR in the respirometry trials was 8.5±0.8 for alate queens, 12.4±0.7 for undug foundresses, and 23.4±0.9 μg H_2O mg^{-1} h^{-1} for dug foundresses.

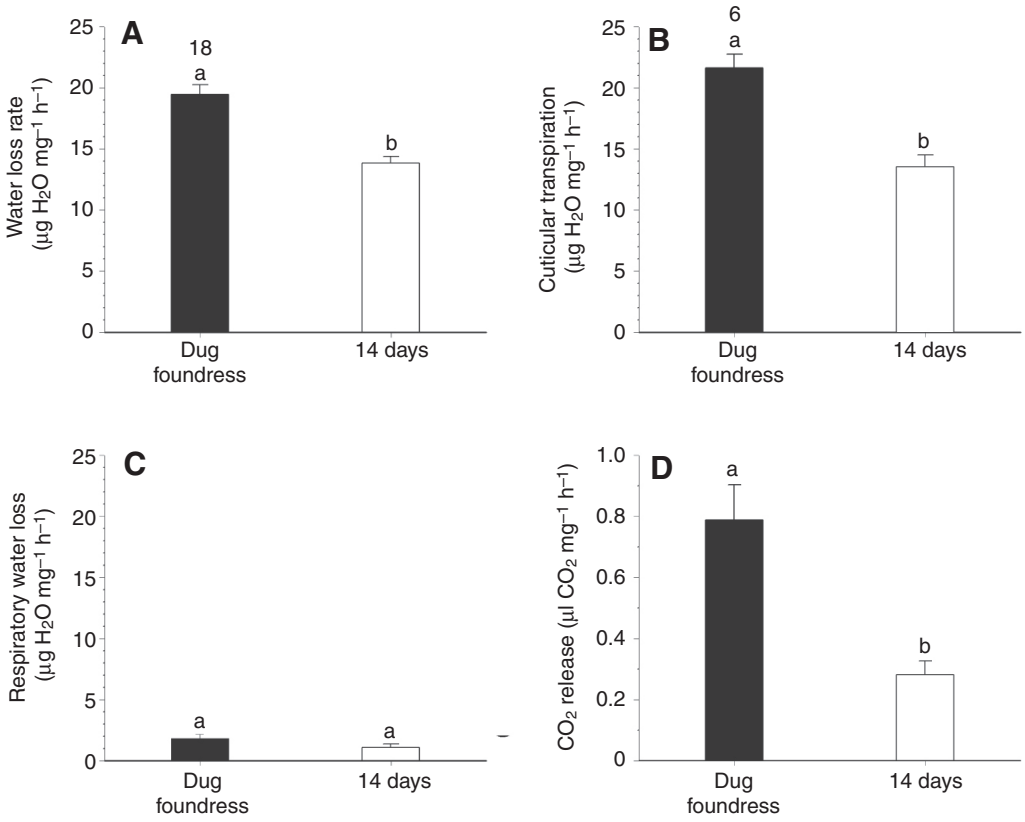

Fig. 7. Effect of a 14-day recovery period on (A) total mass-specific water loss rates (WLRs), (B) cuticular transpiration, (C) respiratory water loss ($\mu g\,H_2O\,mg^{-1}\,h^{-1}$) and (D) carbon dioxide release ($\mu l\,CO_2\,mg^{-1}\,h^{-1}$) for dug foundresses (see text for details). For comparison, total mass-specific WLR in the respirometry trials was 23.4±0.9 for dug foundresses and 14.6±1.0 $\mu g\,H_2O\,mg^{-1}\,h^{-1}$ after the 14-day recovery interval. Significant differences across the two intervals are indicated by the letters a–b: a>b. Sample size is given above each stage; sample size is the same in B–D.

and treatment (Table 2; Fig. 8B). An *a posteriori* one-way ANOVA ($F_{8,54}$=10.56, P<0.001) followed by a Duncan's multiple range test indicated that the emery paper treatment significantly increased cuticular transpiration, to the point that these queens displayed the highest rates of cuticular transpiration among all treatment groups. After the 14-day recovery period, cuticular transpiration decreased significantly in the filter paper and emery paper treatments. The rate of respiratory water loss did not display any significant variation through time (within-subjects effect) or across treatments (between-subjects effect; Table 2; Fig. 8C). Metabolic rate was significantly higher for the control treatment than for the filter paper or emery paper treatment, whereas the within-subjects effect indicated that metabolic rate was significantly lower for the 14-day recovery measurement than for the pre-abrasion or post-abrasion measurements. The interaction term between time and treatment was not significant (Table 2; Fig. 8D). An *a posteriori* one-way ANOVA ($F_{8,54}$=3.9, P<0.001) followed by a Duncan's multiple range test indicated that metabolic rate was higher for untreated queens than for the filter paper and emery paper treatments for all three time intervals.

Our respirometry results suggested a strong correlation between mass-specific cuticular transpiration (CT) and total mass-specific WLR (TWL) in all treatment groups, especially for dug foundresses and artificially abraded alate queens. To determine the significance of this correlation, we regressed CT (independent variable) against TWL (dependent variable) for all individuals with respirometry data;

Table 2. Results of a one-way repeated-measures ANOVA for the effect of treatment and time on mass-specific water loss rates (WLRs) in the artificial abrasion experiment with alate queens of the ant *Messor pergandei*

	Total water loss rate			Cuticular transpiration			Respiratory water loss			Metabolic rate		
Source of variation	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Within subjects[a]												
Time	1.74	18.97	<0.001	1.28	24.12	<0.001	1.19	1.79	0.234	1.48	8.83	0.003
Time×treatment	3.49	7.66	<0.001	2.60	8.60	<0.002	2.38	0.57	0.603	2.97	1.68	0.20
Error	97.65	–	–	23.05	–	–	21.42	–	–	26.68	–	–
Sphericity[b]	2	8.74	0.013	2	14.04	0.001	2	19.38	0.001	2	7.30	0.03
Box's test	12, 7824	0.78	0.67	12, 771.65	0.79	0.666	12, 771.65	1.48	0.124	12, 771.65	1.35	0.186
Between subjects												
Treatment	2	24.94	<0.001	2	1.61	0.227	2	2.01	0.163	2	5.59	0.010
Error	56	–	–	18	–	–	18	–	–	18	–	–

Each variable was run as a separate repeated-measures ANOVA. Total WLR is based on gravimetric data while the other three variables are based on respirometry data.

[a]Within-subjects d.f., *F*- and *P*-values are based on the Greenhouse–Geisser Epsilon value.

[b]In the case of sphericity, approximate χ^2-values are presented instead of *F*-values.

See also Fig. 8.

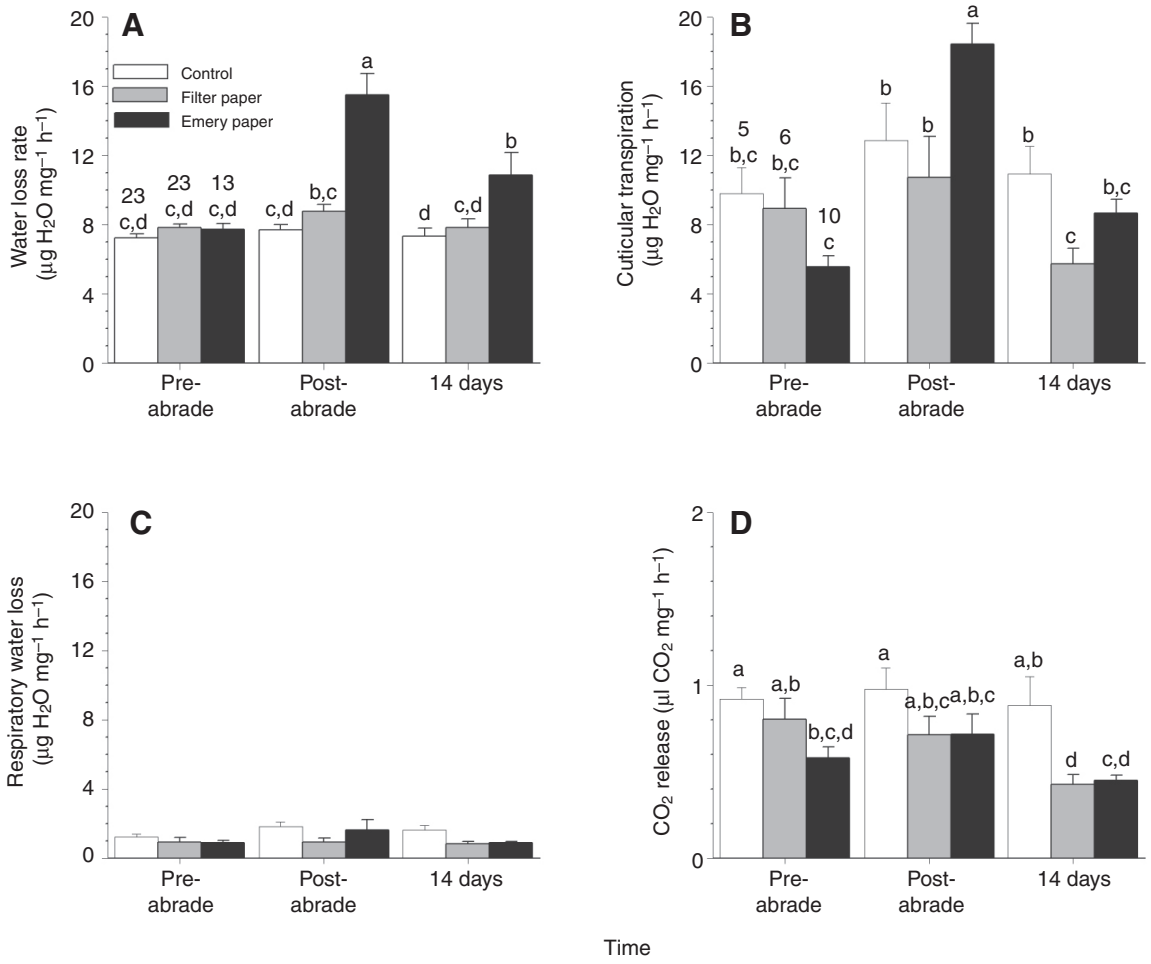

Fig. 8. Effect of artificial abrasion on (A) total mass-specific water loss rates (WLRs), (B) cuticular transpiration, (C) respiratory water loss ($\mu g\,H_2O\,mg^{-1}\,h^{-1}$) and (D) carbon dioxide release ($\mu l\,CO_2\,mg^{-1}\,h^{-1}$) in alate queens. Alate queens were tested at three intervals; after removal from their natal nest (pre-abrade), within 24–72 h of the experimental treatment (post-abrade) and 14 days after treatment (14 days). Results for the repeated-measures ANOVA are given in Table 2. Significant differences within each panel are denoted by the letters *a–d*: *a>b>c>d*. Sample size is given above each stage; sample size is the same in B–D.

the slope of the regression indicates the proportionate increase in TWL that occurs through the cuticle (CT). One outlier was omitted from the analysis. Across all individuals, CT was positively correlated with TWL [TWL=1.039 (±0.015) × CT + 0.417 (±0.199), N=64, $F_{1,62}$=4819.0, R^2=0.987 P<0.00001; Fig. 9]. The slope of the regression (1.039) indicates that CT accounted for ~104% [lower 95% confidence interval = 1.016] of the increase in the TWL associated with excavating a nest. Analysis of metabolic rate and respiratory water loss (RWL) corroborated these results. Using a stepwise multiple regression, both CT (P<0.0001) and RWL (P<0.0001) were correlated with TWL. CT accounted for 99.4% of the variation in TWL, and RWL only accounted for an additional 0.2%. Metabolic rate was also included in the model (P=0.036), but it did not explain additional variance related to TWL. In a separate regression, RWL correlated with metabolic rate [$F_{1,62}$=25.3, R^2=0.29, P<<0.001; RWL=5.970 (±1.186) × metabolic rate – 1.269].

DISCUSSION

Potential mechanisms for increased cuticular water loss

Mating and colony founding by queen *M. pergandei* involve a series of activities that include: (1) queens and males flying from the nest, (2) mating in the air with *in copulo* pairs sometimes falling to the ground, (3) mated queens removing their wings, and (4) queens excavating nests and beginning to lay eggs. Three mechanisms could increase cuticular transpiration along this sequence: (1) wing loss by mated queens, (2) changes in the amount and/or composition of cuticular hydrocarbons and (3) abrasion that scratches and/or displaces the protective layers of the cuticle. In the third mechanism, abrasion might occur by mechanical damage during mating and/or during the process of nest excavation. We evaluate these mechanisms using our experimental data and by comparing WLRs of live and dead queens, assuming that direct support for one of the proposed mechanisms requires increased WLRs regardless of vital state.

The hypothesis that nest excavation abrades the cuticle and increases WLRs for dug foundresses is supported by several lines of correlative and experimental data. The significantly higher WLRs and higher levels of cuticle damage on dug foundresses compared with alate queens provided strong correlative evidence that abrasion increases WLRs. That the changes in water loss were not caused by regulatory processes of the living foundresses were confirmed by the increased WLRs for both live and dead dug foundresses. We were able to provide direct experimental support for cuticle abrasion as the cause of increased WLRs, because

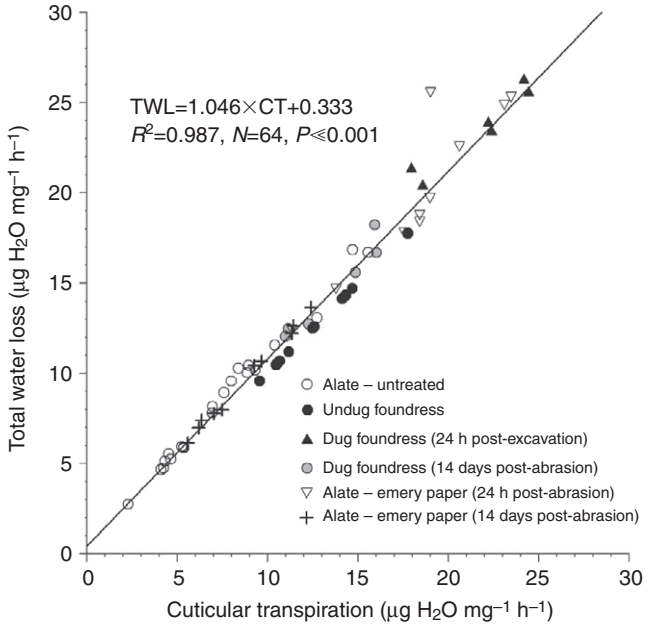

Fig. 9. Relationship between cuticular transpiration (CT) and total water loss (TWL) (μg H_2O mg^{-1} h^{-1}) in queens at the three mating stages. Symbols denote queen status (see text for details). The outlier at the top left of the regression line was omitted from the analysis (see text).

artificial abrasion increased WLRs of alate queens to levels as high as or higher than those for dug foundresses (Figs 5, 8), whereas WLRs were unaffected in the control groups. Lastly, respirometry data identified that essentially all the increased water loss of undug foundresses, dug foundresses and artificially abraded queens resulted from increased cuticular transpiration (Fig. 9). Cuticle abrasion might also occur through mechanical damage during mating, but this hypothesis is not supported, given that WLRs increased in live but not dead undug foundresses (Fig. 5).

We also reject other potential mechanisms. First, the hypothesis that water escapes through a temporary opening after undug foundresses tear off their wings is rejected because WLRs following wing loss increased for live but not for dead undug foundresses (see also Johnson, 2000c). Second, although cuticular lipids were not assayed during this study, we believe the hypothesis that changes in the amount and/or type of cuticular lipids increase water loss is unlikely because the total quantity of hydrocarbons did not differ by mating stage for another seed-harvester ant, *Pogonomyrmex barbatus*, either on an individual basis or per unit surface area (Johnson and Gibbs, 2004). The relative composition of hydrocarbons did vary significantly across mating stages for queens of *P. barbatus*, but the overall changes were small, and it was judged that these levels of change would not increase WLRs by between twofold and threefold (Johnson and Gibbs, 2004). Third, we note that age does not cause any of the observed differences in WLRs across mating stages because the queens used in our experiments were produced as a cohort, and individuals from all three mating stages were collected and tested over the same time interval. Lastly, metabolic rate and activity did not vary across mating stages, ruling out these mechanisms as an explanation for increased WLRs in dug foundresses (Fig. 6D).

Previous studies that have examined cuticle abrasion typically abraded insects with inert dusts such as silica or aluminum powder (Collins, 1969; Hafez et al., 1970; Holdgate and Seal, 1956; Nel, 1965; Wigglesworth, 1945). However, these inert dusts are problematical for documenting cuticle abrasion because particles could enter and damage tracheal tubes, and/or interfere with functioning of the spiracular valve. Most of these dusts abrade the cuticle, but they also absorb epicuticular waxes [Ebeling (Ebeling, 1971) and references therein], which can contribute to increased WLRs. Moreover, we assert that inert dusts should not be used in cuticle abrasion studies because they poorly mimic conditions experienced by soil-digging insects. Alternatively, the emery paper used in our artificial abrasion experiment was a quick and easy method to abrade individuals, and its action appeared to mimic natural conditions given that the head and anterior dorsal portions of the mesosoma have the most contact with soil particles during nest excavation. Interestingly, lightly rubbing alate queens with emery paper 10 times on the head and mesosoma resulted in similar WLRs to those of dug foundresses.

Recovery from cuticle abrasion

Both dug foundresses and artificially abraded alate queens showed partial recovery from cuticle abrasion after 14 days, whereas untreated alate queens did not change over the same interval. As predicted by the cuticle abrasion hypothesis, recovery in both groups of queens resulted in a significant decrease in cuticular transpiration, but not in respiratory water loss. Interestingly, both types of queens exhibited a similar degree of recovery after 14 days (29.5% decrease in total WLRs for dug foundresses, 30.0% decrease for artificially abraded alate queens), again suggesting that our treatment with emery paper mimicked natural abrasion.

Several other studies document recovery following abrasion by inert dusts, but none of these studies included respirometry data or examined recovery of field-collected individuals (Hafez et al., 1970; Machin and Lampert, 1987; Wigglesworth, 1945; Wolfe, 1955). All these studies also noted that most recovery occurred within 3 days, but that individuals rarely recovered completely (Hafez et al., 1970; Wigglesworth, 1945). Another study on cockroaches suggested that crowding caused cuticle damage because WLRs for individuals maintained in groups were significantly higher than those for individuals that had been isolated for 3 days; cuticular recovery was presumed to cause the lower WLRs for isolated individuals (Machin and Lampert, 1987). Our study did not examine the time course of recovery, but rather we documented that recovery occurred and that it was mostly incomplete after 14 days. By that time, mass-specific WLRs for both field-collected and artificially abraded queen *M. pergandei* had decreased by ~30%.

The mechanism leading to recovery is unknown, but it probably relates to production of cuticular hydrocarbons that are transported through wax channels to the surface of the cuticle. These lipids then presumably reseal the cuticle by spreading out to fill in the scratches and furrows caused by abrasion (Lockey, 1988; Schal et al., 1998; Wigglesworth, 1945). The ability of insects to recover from abrasion probably depends on the amount of damage incurred and whether damage is restricted to surface layers or if it also extends to the cuticle (see Wigglesworth, 1937). In the field, recovery by dug foundresses will probably be slowed by continued contact with soil particles while moving through nest tunnels. Such continued contact with soil particles probably also slows recovery in other soil-dwelling insects.

Cuticular transpiration, respiratory water loss and metabolic rate

WLRs for queens of *M. pergandei* increased dramatically from the alate to dug foundress stage, which corresponds to the pattern

observed in previous studies on *M. pergandei* and other species of desert ants (Johnson, 2000c; Johnson and Gibbs, 2004). Our regression method identified that cuticular transpiration was the primary avenue of water loss for queen *M. pergandei*, accounting for an average of 90.6% (range=81.7–99.6%) of the total water loss across all individuals. Additionally, increased cuticular transpiration accounted for essentially all (an estimated 104%) of the increase in total water loss displayed by undug foundresses, dug foundresses and artificially abraded queens relative to that of alate queens (see Fig. 9). Respiratory water loss and metabolic rate also contributed to total water loss in these queens. However, after including cuticular transpiration in our stepwise multiple regression model, the effect of respiratory water loss and metabolic rate were minimal as both variables together only explained ~0.2% additional variance in the model. Additionally, activity levels were similar across treatments in all our experiments, indicating that variation in activity did not influence our results.

The technique of regressing water loss rate against CO_2 release rate is one of three methods that have been used to estimate respiratory water loss. The traditional discontinuous gas-exchange cycle method, where cuticular water loss rate is determined in phases where spiracles are closed and CO_2 release is minimal, can only be used for insects that display discontinuous gas exchange (Gray and Chown, 2008), which was not the case for most of our *Messor* queens. The other technique relies on spiracular closure by the insect following a manipulative exposure to hyperoxia (Lighton et al., 2004; Schilman et al., 2005). Each of the latter two methods has advantages and disadvantages. The hyperoxia method appears to overestimate respiratory water loss and requires manipulating the organism (Gray and Chown, 2008). Alternatively, the regression method provides a means of estimating respiratory water loss for all types of ventilation, but has a number of drawbacks: (1) it extrapolates values to the *y*-intercept and thus assumes linearity when insects do not fully close their spiracles, (2) the regression occasionally results in a negative *y*-intercept, (3) the data points are autocorrelated and (4) it is more sensitive to the different flow-through kinetics of CO_2 and water vapor (Chown et al., 2006; Gray and Chown, 2008; Schilman et al., 2005). Nevertheless, the regression method generates repeatable results for estimating cuticular transpiration in the absence of spiracular closure without the need for manipulation (Chown et al., 2006; Gray and Chown, 2008). Moreover, we used the regression method so as to avoid potentially confounding manipulations other than those that were used in our experimental design.

Both dug foundresses (Fig. 7) and alate queens in the abrasion experiment (Fig. 8) displayed a significant decline in metabolic rate after 14 days in the laboratory. In other arthropods, such declines in metabolic rates have been attributed to acclimation that is caused by factors such as reduced stress or decreased temperature variability in the laboratory (Terblanche et al., 2004; Terblanche et al., 2007). The ecology of ant queens provides an alternative explanation for the decreased metabolic rate. Alate queens in their natal nest and recently captured foundresses are likely to metabolize carbohydrate reserves, whereas several days after mating flights the foundresses are likely to sustain themselves by metabolizing fat reserves (Johnson, 1998), and this switch results in the respiratory quotient decreasing from 1.0 to 0.71. If this switch occurred in our queens, then CO_2 release rates, and hence the estimated metabolic rate, would have decreased by ~30% after the 14-day recovery interval, even though the actual metabolic rate had not changed. Such a shift of metabolic substrate would also explain why respiratory water loss did not decrease in concert with metabolic rate for either group of queens.

Some water loss trials ran for less than 8 h. Direct comparison of these trials with those that ran for 8 h assumes that water loss is linear throughout our test period. We tested this assumption by measuring water loss for 29 alate queens at 1, 2, 4 and 8 h. Data for each interval were standardized as mass loss, in $\mu g\,h^{-1}$, then compared across the four time intervals using a one-way ANOVA. Results showed that rate of water loss was similar across the four time intervals (one-way ANOVA: $F_{3,112}=1.64$, $P>0.18$), thus verifying our assumption of linear water loss during the 8 h interval. Additionally, all queens were fully hydrated before their initial WLR trial, and individuals regained most of the water lost during trials (~50%) within 24 h, which was the minimum interval between trials.

Two of the data sets had anomalous results – gravimetric data showed lower total WLRs for undug foundresses than for alate queens (Fig. 6A), and respirometry data showed a higher respiratory water loss rate for dug foundresses compared with alate queens and undug foundresses (Fig. 6C). It is unclear why our gravimetric total WLRs were lower for undug foundresses than for alate queens. Moreover, we doubt the validity of these data because respirometry data on a subset of these individuals showed that WLRs for undug foundresses were intermediate to those of alate queens and dug foundresses; this latter pattern also matches that found in the harvester ant *P. barbatus* (Johnson and Gibbs, 2004). Despite this discrepancy we were able to demonstrate that the greatest increase in WLRs resulted from nest excavation rather than from mating and tearing off their wings. For the second data set, the higher respiratory water loss rates for dug foundresses (N=6) than for alate queens and undug foundresses resulted from one outlier: omitting this individual changed the P-value in the one-way ANOVA from 0.007 to 0.09, such that respiratory water loss rate was then similar across the three mating stages.

Conclusions

This study links water loss in natural habitats to causative mechanisms for *M. pergandei* queens, thus providing evidence that ecologically relevant physical damage to the cuticle can have a significant effect on water balance. Comparing WLRs and cuticular damage on the same individuals, combined with our artificial abrasion experiment, provided a powerful technique to assess the reasons for variation in WLRs across mating stages. This study also demonstrates the need to consider cuticle condition when assessing intra- and inter-specific variation in WLRs, habitat variation in WLRs, or when conducting studies on the evolution of WLRs and desiccation resistance.

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