# A cross-species test of the function of cuticular traits in ants (Hymenoptera: Formicidae)

James T. Buxton, Kylie A. Robert, Alan T. Marshall, Travis L. Dutka & Heloise Gibb

### Abstract

The trait-based approach to ecology promises greater generality in our understanding of how species assemblages are structured. The value of this approach depends on linking traits with ecological functions, but no studies have tested the proposed functional values of many commonly used and easily measured traits. We used a cross-species comparison to investigate the function of the cuticular traits "pilosity" and "sculpturing" in ants. These traits have been linked to a range of environmental variables through a correlative approach, but the mechanisms underlying these associations are poorly understood. Here, we tested the association among these traits and with a suite of physiological traits (heat tolerance, desiccation tolerance, hydrophobicity and protection from physical forces). Further, we tested whether physiological traits differed between species that were active at day or night or ants from semi-arid and mesic environments. Many cuticle-related traits were inter-correlated and not independent of other morphological traits, suggesting that it is appropriate to consider ant responses as an "ecological strategy". Although within-assemblage variation was high, night-active and mesic ants had lower heat tolerance than day-active and semi-arid ants, reflecting similar differences in environmental exposure. Pilosity was associated with both heat and desiccation tolerance. Sculpturing was strongly linked to cuticle thickness and hardness and desiccation tolerance. However, it was negatively associated with heat tolerance, possibly because many highly sculptured ants were nocturnal in this study. This work provides strong support for the continued use of easily measured cuticle traits. However, empirical investigations of trait function across a broader range of ecosystems are critical to ensure that the ecological importance of trait-environment relationships and their inter-relatedness as components of an "ecological strategy" are better understood.

**Key words:** $CT_{max}$, desiccation tolerance, functional traits, morphological traits, pilosity, sculpturing, trait-environment relationships, trait-based ecology.

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## Introduction

The development of general rules explaining the spatial and temporal structure of species assemblages has been identified as the central challenge of community ecology (Lewontin 1974, Keddy 1992, Simberloff 2004). The failure to develop such rules limits our ability to understand and ameliorate reductions in biodiversity and ecosystem function in response to global change (McGill & al. 2006, Cardoso & al. 2011, Andrew & al. 2013). Recording the occurrence of species traits of known functional importance and linking them with environmental variables has been touted as a way to understand the structure of species-assemblages in a mechanistic way, allowing patterns in assemblage structure to be predicted on a global scale (McGill & al. 2006, Violle & al. 2007, Messier & al. 2010, Webb & al. 2010, Verberk & al. 2013, Fountain-Jones & al. 2015). This trait-based approach to ecology works under the assumption that the pool of species traits available are the result of selection based on function that is then "filtered" by abiotic and biotic environmental factors (Kraft & al. 2007, Webb & al. 2010, Weiher & al. 2011).

Morphological traits are informative for investigating species-environment interactions if they are easily measured and of functional importance (Yates & al. 2014, Gibb & al. 2015b, Parr & al. 2017). They are likely to be especially effective for arthropod communities in which a large proportion of species may be poorly known taxonomically and ecologically, but can be rapidly assessed

Tab. 1: The hypothesised function of pilosity and sculpturing-related traits.

| Hypothesised function | Justification | Reference |
|---|---|---|
| **Pilosity-related** | | |
| Defence against predation | Certain specialised hairs may serve a defensive function. Higher pilosity is associated with predatory ants, suggesting that pilosity may provide physical protection. | (Gnatzy & Maschwitz 2006, Gibb & al. 2015b) |
| Prevent water immersion | Hydrophobic hairs may be an adaptation to prevent immersion of the cuticle in water and/or escape from water droplets or bodies of water. | (Pal 1950, Suter & al. 2004, Bush & al. 2008) |
| Desiccation tolerance | In studies of other arthropods pilosity has been found to reduce water loss. By reducing disturbance to the air boundary layer above the cuticle, even sparsely distributed hairs may serve to increase boundary layer resistance to water loss. Hydrophobic hairs situated over spiracles may reduce water loss. | (Wolpert 1962, Wuenscher 1970, Casey & Hegel 1981, Kevan & al. 1982) |
| Thermoregulation | Hairs may protect the warm boundary layer from disturbance, allowing absorbed heat to be retained by reducing convective heat exchange. Foliage-dwelling spiders had a higher pilosity at lower temperatures suggesting a possible thermoregulatory function. Hair reflectivity can influence body temperatures, and hair fields may increase UV resistance. | (Wolpert 1962, Heinrich 1974, Casey & Hegel 1981, Kevan & al. 1982, Fields & McNeil 1988, Gibb & al. 2015a, Shi & al. 2015, Peters & al. 2016, Willot & al. 2016) |
| **Sculpturing-related** | | |
| Structural protection (including defence) | A "more structured" cuticle is considered a defensive adaptation. A greater degree of sculpturing has been linked to a lower C:N ratio, suggesting ants with a high degree of sculpturing possess a harder, thicker cuticle. The amplitude, frequency, and area of sculpturing units could be expected to be influential for the geometrical strain-hardening and/or compressive strength of cuticles, both for irregular hexagonal cells and corrugations. | (Hunt 1983, Vincent & Wegst 2004, Gibson 2005, Alkhader & Vural 2008, Gibb & al. 2015b) |
| Desiccation tolerance | By increasing the thickness of the air boundary layer above the cuticle sculpturing may serve to increase boundary layer resistance to water loss. A thicker cuticle may reduce water loss in ants. | (Wolpert 1962, Wuenscher 1970, Chen & al. 2014, Bishop & al. 2016) |
| Thermoregulation | By increasing the cuticle surface area sculpturing would be expected to provide increased convective heat loss from the cuticle surface through the boundary layer. A thicker cuticle may contribute to heat and cold tolerance in ants. The structure of the cuticle may also influence solar radiation absorption. | (Wolpert 1962, Galushko & al. 2005, Plotkin & al. 2010, Chen & al. 2014) |

morphologically (Kaspari & Weiser 1999, Pollock & al. 2012, Yates & al. 2014). The investigation of morphological trait-environment relationships has a long history in land plants (Darwin 1877, Grime 1977, Ackerly & al. 2002, Pollock & al. 2012) and has recently received attention for arthropod (Babin-Fenske & al. 2008, Eweleit & Reinhold 2014, Gibb & al. 2015a, Gibb & al. 2015b, Brousseau & al. 2018, Wong & al. 2019), vertebrate (Dell & al. 2011, Garcia & al. 2014) and microbial assemblages (Green & al. 2008). There has been some success in linking traits with environmental variables on both local and global scales (Kaspari 1993, Gibb & Parr 2013). However, many "functional traits" might be correlated with other unmeasured traits, be the result of trade-offs among traits (Nijhout & Emlen 1998, Verberk & al. 2013, Shiel & al. 2015), or even be Gould & Lewontin (1979)'s functionless biological "spandrels". Empirical studies linking traits with their functions are an essential component in developing a cohesive research program for the trait-based approach (Mlambo 2014, Fountain-Jones & al. 2015).

Across the globe, environments vary immensely in their physical and biological conditions, but much of the variation experienced by organisms occurs within single locations, due to diurnal or seasonal differences or differences among microhabitats (Kaspari & al. 2015). As a consequence of this small-scale variation, species may vary substantially in their traits within local communities. For example, nocturnal and diurnal Lepidopteran species differ substantially in a range of key sensory and physiological traits (Lyytinen & al. 2004, Gaston 2019).

Similarly, species that occupy different components of the same environment, such as canopy branches and leaf litter, show immense physiological differences: Kaspari & al. (2015) found that the critical thermal maxima ($CT_{max}$) of ant species from Panama rainforest occupied 74% of the total global variation in $CT_{max}$ for ants. Similarly, T.R. Bishop (unpubl.), showed that ant assemblages from single localities occupied up to 80% of global morphospace. However, between-site variation can also be significant, for example, even within the same species, physiological and morphological traits differ substantially along latitudinal and elevational gradients (Oyen & al. 2016, Bishop & al. 2017, Shik & al. 2019).

Ants (Hymenoptera: Formicidae) are particularly suitable study organisms for the trait-based approach due to their ubiquitous nature and high biomass (Fittkau & Klinge 1973), their important ecological roles (Andersen 1988, Hölldobler & Wilson 1990, Sanders & van Veen 2011, Del Toro & al. 2012), and their great morphological variation (Wheeler 1910, Weiser & Kaspari 2006, Yates & al. 2014, Gibb & al. 2015b). Whilst the use of the trait-based approach in myrmecology is still in its infancy, strong links to environmental variables have been discovered for a large number of morphological, physiological and ecological species traits (Silva & Brandão 2010, Wiescher & al. 2012, Gibb & Parr 2013, Yates & al. 2014, Gibb & al. 2015b), and new techniques for analysing relationships have been developed (Brown & al. 2014, Stoklosa & al. 2014). However, for many of these traits, empirical investigations into function have not been undertaken and, as a result, our ability to interpret the trait-environment relationship is limited.

Cuticle traits are likely to be important in a range of functions, including heat and desiccation tolerance, hydrophobicity and defence (Tab. 1). "Pilosity" and "sculpturing" are morphological cuticle traits that are easily observed under a dissecting microscope (Gibb & al. 2015b). "Pilosity" refers to the density of any erect setae (hairs) on the mesosoma (Parr & al. 2017), rather than pubescence (shorter, prostrate setae), fine hair fields between joints, or large bristles on the tibia. Pilosity has been associated with sensory traits, predatory behaviour and open areas, suggesting a possible sensory, defensive or physiological function (such as protection from desiccation), respectively (Yates & al. 2014, Gibb & al. 2015a). "Sculpturing" refers to depressions and ridges on the integument. In trait-based myrmecological studies, sculpturing is measured using a coarse ordinal score of 1 to 3 based on the perceived "degree" of sculpturing (relative measure based on a combination of sculpturing characteristics such as area, frequency, and particularly depth of sculpturing, Parr & al. 2017). Sculpturing has been shown to be greater in predatory species, suggesting a defensive function (Gibb & al. 2015b). Despite evidence that these cuticle traits correlate with environmental variables and adoption as key traits in the Global Ants Database (Gibb & al. 2017, Parr & al. 2017), the functional significance of pilosity and sculpturing remains untested.

We used a cross-species comparative approach to test the functions of pilosity and sculpturing and related cuticle traits in ants (Tab. 1). We ignored intraspecific variation because it makes only a small contribution (1 - 4%) to total trait variation (Gaudard & al. 2019). Diurnal and nocturnal species were collected from a semi-arid and a mesic environment to obtain a broad range of morphologies. We asked: 1) How are pilosity and sculpturing traits related to each other?; 2) What are the key axes of variation among species traits relating to cuticle structures?; 3) How do activity, location and subfamily affect the position of species along key axes of variation?; and 4) How do pilosity and sculpturing (or cuticle thickness) affect physiological traits, including $CT_{max}$, mass-independent water loss, puncture force and adhesion energy? We expected that diurnal ants and those from the semi-arid environment would have greater temperature and desiccation tolerance (reflected in higher $CT_{max}$, lower mass-independent water loss and greater adhesion energy) than nocturnal ants and ants dwelling in a more mesic environment and that physiological traits would differ among subfamilies. We predicted that increased sculpturing and pilosity would indicate a stronger barrier against microclimates and therefore be associated with increased tolerance to temperature and desiccation and increased adhesion energy (which indicates decreased hydrophobicity or increased wettability). Further, we predicted that more sculptured cuticles would require greater force to puncture.

**Materials and methods**

**Field sites:** Field work was conducted at Scotia Sanctuary (semi-arid; 33° 21' S, 141° 17' E) for seven days and three nights from 22 - 28 October 2014, and La Trobe Wildlife Sanctuary (mesic; 37° 43' S, 145° 43' E) for seven days and three nights from 9 - 23 December 2014. Scotia Sanctuary is a 64,000 ha property on the arid / semi-arid climate boundary of Western NSW, Australia, experiencing irregular rainfall (annual average 250 mm), hot summers, and cool winters. Temperature measured approximately 1.5 cm from the ground at Scotia ranged from 18.3 °C to 40.5 °C and humidity from 13.1% to 68.1% during the sampling period. All collecting at Scotia was conducted in open spinifex-mallee (*Triodia scariosa* Burbidge, 1953) understory with overstory dominated by *Eucalyptus socialis* Mueller ex Miquel, 1826, *Eucalyptus dumosa* Cunningham ex Oxley, 1820 *and Eucalyptus gracilis* Mueller, 1855 or *Casuarina pauper* Mueller ex Johnson, 1989 woodland vegetation.

La Trobe Wildlife Sanctuary is a 28 ha reserve on the La Trobe University campus in Melbourne in South-Eastern Australia, containing both revegetated and remnant river red gum (*E. camaldulensis* Dehnhardt, 1832) woodland with a grassy understory and having a mean annual rainfall of 659.8 mm. Temperature measured approximately 1.5 cm from the ground ranged from 17.0 °C to 31.5 °C and humidity 21.3% to 82% during the sampling period. Ants were collected in a variety of microhabitats within semi-arid and mesic environments, and both night (20:00 - 02:00 h)

Tab. 2: The morphological traits used in this study with descriptions. Justifications are provided for all traits other than pilosity and sculpturing-related traits.

| Morphological trait | Justification | Reference |
|---|---|---|
| **Size-related** | | |
| Weber's length | Distance from the anterodorsal margin of the pronotum to the posteroventral margin of the propodeum (mm). Indicative of worker body size. | (WEBER 1938) |
| Pronotum width | Maximum distance across the middle of the pronotum, ignoring tubercles (mm). Indicative of body mass and reflects the size of cuticles used for the puncture force experiment. The likelihood of compressive failure and buckling should increase as the thickness to size ratio of a piece of cuticle decrease. | (KASPARI & WEISER 1999, VINCENT & WEGST 2004) |
| Eye length | Measured across the maximum length of the eye (mm). Indicative of food searching behaviour and activity times. | (WEISER & KASPARI 2006) |
| Scape length | Length of antennal scape (mm). Related to both mechano-reception and chemoreception abilities. Longer scapes facilitate following of pheromone trails. | (WEISER & KASPARI 2006) |
| **Pilosity-related** | | |
| Pilosity | Count of hairs crossing the dorsal surface of mesosoma in lateral view. | (PARR & al. 2017) |
| Greatest hair length | Longest hair on dorsal surface of the mesosoma (mm). | (YATES & al. 2014) |
| Pubescence score | Ordinal score out of three (1 = absent, 2 = sparse, 3 = dense). | |
| Hair colour score | Ordinal score out of three (ordinal, 1-3, 1 = white, 2 = yellow, 3 = dark brown). | |
| **Sculpturing-related** | | |
| Sculpturing score | Ordinal score out of three. Scored on the pronotum, mesonotum, and propodeum. The "overall sculpturing score" as the degree of sculpturing across the entire mesosoma was also scored (1 = no depressions or ridges, 2 = moderately deep depressions and ridges, 3 = deep sculpturing that covers a large area). | (PARR & al. 2017) |
| Amplitude score | Not previously utilised in trait-ecology studies. The relative "depth" of sculpturing. Ordinal score out of three (1 = flat, 2 = moderately deep, 3 = very deep) | |
| Freq. sculpturing units | Not previously utilised in trait-ecology studies. The number of discrete sculpturing units on pronotum per mm. | |
| Area sculpturing units | Not previously utilised in trait-ecology studies. The mean area of discrete sculpturing units on pronotum per mm. Negatively related to frequency of sculpturing units. | |
| Proportion depressions | Not previously utilised in trait-ecology studies. The proportion of the total pronotum area (pronotum length × width) taken up by depressed areas. | |
| Proportion ridges | Not previously utilised in trait-ecology studies. The proportion of the total pronotum area (pronotum length × width) taken up by raised areas. | |

and day (08:00 - 18:00h) sampling was conducted to ensure a sufficiently broad suite of species was collected to test predictions.

**Ant sampling:** Foraging workers were hand-collected in two hour collecting periods during the day and at night to capture diurnally and nocturnally active species. Temperature, humidity and, habitat complexity were recorded at all collecting sites. Ten ants were collected per monomorphic species and 16 per polymorphic species, where possible. When collected, ants were first stored in plastic jars with a damp cotton bud before being put through a desiccation or thermoregulation experiment within three hours of being collected. Ants were identified to genus using SHATTUCK (1999) and then to "morphospecies" (OLIVER & BEATTIE 1996) in the field. Morphospecies were later identified to species where possible, using the reference collection at La Trobe University. Reference specimens are held at La Trobe University.

**Trait measures:** Fourteen morphological traits were measured for five individuals of every morphospecies,

where possible (Tab. 2). Traits related to pilosity and sculpturing were selected based on their use in the available literature or hypothesised importance. Size traits were selected based on known relationships between size and physiological responses, and sensory traits were selected based on established links and possible trade-offs with pilosity (Tab. 2). Measurements were made using the live measurement function (Leica Application Suite 3.8.0 Leica Microsystems Ltd., Wetzlar Germany) with a Leica M165C microscope (Leica Microsystems Ltd., Wetzlar Germany). All continuous sculpturing measures were measured on images of the pronotum using Fiji version 2.0. (Schindelin & al. 2012). Sculpturing measures were restricted to the dominant sculpturing type; the presence of other sculpturing was recorded qualitatively but not included in analyses.

**Desiccation experiment:** A desiccation experiment was used to determine the influence of traits on the rate of water loss of ant species using five ants per monomorphic species and eight per polymorphic species, where possible. Ants were put in a "chill coma" (frozen at approximately -18 °C for five minutes to immobilise them for one to five minutes) before taking initial mass readings (mg) on a microbalance (XS3DU Mettler Toledo, Greifensee, Switzerland). All individuals were then deposited into plastic vials with perforated lids, which were placed in a plastic desiccator (3.5 L) with a 2 cm layer of 3A molecular sieves in a room of stable temperature and humidity (Scotia average temperature ± standard deviation 24.6 ± 1.4 °C, average humidity 38.3 ± 8.7%; LTU temperature 24.0 ± 0.4 °C, humidity 47.8 ± 5.6%) for two hours at a low relative humidity (near 0%). After two hours, ants were again put into a chill coma and weighed. Mass loss rate is considered proportional to water loss rate in ants as the contribution of respiratory $CO_2$ emission to water loss has been found to be negligible (Lighton & Feener 1989). The water loss rate (WLR) was calculated as the change in mass per hour:

$$\text{WLR (µg h}^{-1}) = \frac{\text{initial mass (µg)} - \text{final mass (µg)}}{\text{desiccation duration (h)}}$$

Afterwards, specimens were euthanised by freezing at -18 °C for 20 minutes before being put in an oven at 60 °C for 72 hours (or until they reached a constant mass) and weighed to provide a dry mass. The total water content as a proportion of the entire body mass (PWC) was calculated as:

$$\text{PWC (mg)} = \frac{\text{initial mass (mg)} - \text{dry mass (mg)}}{\text{initial mass (mg)}}$$

The mass-independent water loss rate (MIWLR) was calculated (as in Chen & al. 2014) as:

$$\text{MIWLR (µg h}^{-1}\text{ mg}^{-1}) = \frac{\text{WLR (µg h}^{-1})}{\text{initial mass (mg)}}$$

**$CT_{max}$ experiment:** To determine the relationship between traits and heat stress, the critical thermal maximum ($CT_{max}$) of ants was determined using five individuals per monomorphic species and eight per polymorphic species, where possible. The ants were first put into flat-bottomed shell tubes stopped with a biodegradable packing peanut (one individual per tube) and then placed into the wells of a Stuart block heater (SBH130D, Bibby Scientific Ltd., Staffordshire, UK) in a random order in a room of stable temperature and humidity (average room temperature and humidity equal to the desiccation experiments). The initial temperature of the heating block was 30 °C and was increased in intervals of 2 °C. The ants were given three minutes at each temperature to acclimate. The ants were removed when moribund (not showing a righting response when the tube was turned, as in Diamond & al. 2012).

**Cuticle thickness:** To determine the thickness of the cuticle, the pronotum of frozen ants was sectioned with a sharp razor blade, then stored specimens in 70% ethanol for three to four days, before being dried in hexamethyldisilazane (HMDS) (as described in Bray & al. 1993). Specimens were mounted vertically (sectioned surface upwards) on strips of double-sided carbon tape and further cemented with conductive carbon / graphite paint when necessary. Specimens were coated with 6 nm (60 angstroms) of platinum in a sputter coater (Polaron SC7640, Quorum Technologies Ltd., Newhaven, UK). They were then viewed with a field-emitting scanning electron microscope (FESEM, JSM-634OF, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 2Kv, and measured at five separate points on the pronotum so a mean cuticle thickness could be calculated (Fig. S1, available as digital supplementary material to this article, at the journal's web pages). Where sections were not clearly straight, a stereo image was produced for a 3D representation (Fig. S2) to examine the angle of the cut to determine if an accurate thickness measure could be taken.

**Puncture force:** A puncture force experiment was used to compare the relative hardness of cuticles. Hardness was defined as the peak force before cuticle failure, rather than the definition commonly used in materials science (Freeman & Lemen 2007). The dorsal surface of the pronotum cuticle was excised from frozen specimens using Vannas Micro Dissecting Spring Scissors (RS-5641, Roboz Surgical Instrument Co., Inc., Gaithersburg, USA) and cut into a square shape. The force to penetrate the cuticle was recorded using a Powerlab 26T system (ADinstruments Ltd., Dunedin, New Zealand) with a strain force gauge-based force transducer (MLT5001A, ADinstruments Ltd., Dunedin, New Zealand) clamped to an adjustable stand onto which a headless micropin (15 mm length x 0.193 mm diameter) was attached. A piece of scotch tape was placed upside down on a plastic Petri dish with a 2.5 mm hole attached to a jack. The sample was placed on the tape over the hole and the force to penetrate it measured (in mN). The force to penetrate the cuticle was defined by the maximum force before an obvious, rapid drop in the resistance of the sample (Fig. S3). The amount of force required to pierce the scotch tape (mean ± standard error, SE: 1.8 ± 0.7 mN) was deducted from the total force recorded (mean ± SE: 505.6 ± 64.3 mN). Force was analysed using the program
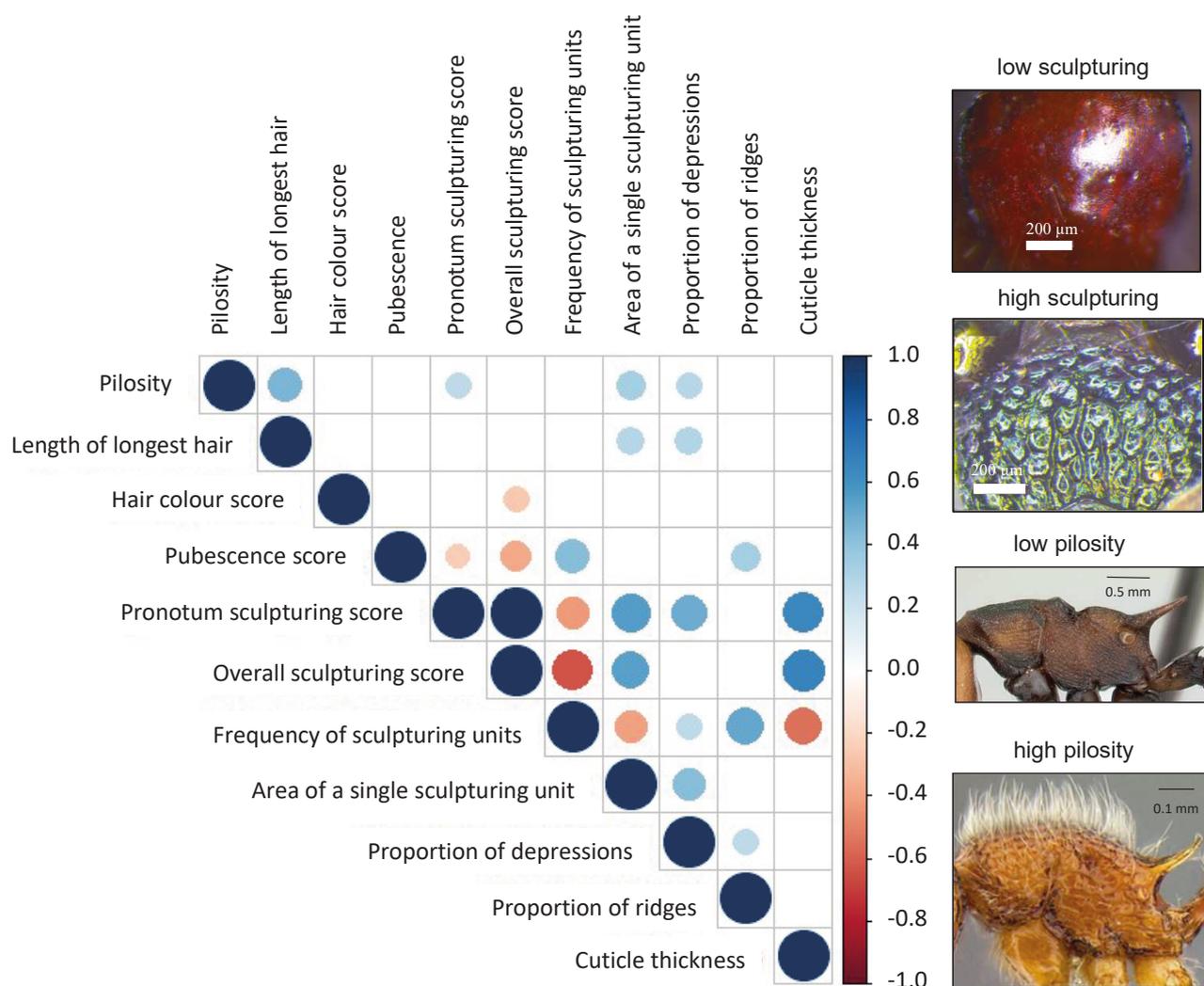
Fig. 1: Correlations among mean cuticle traits for 70 ant species. Circles indicate significant correlations; circle size and colour indicate size of correlation coefficient; positive correlations are shown in blue and negative in red. Inset images show ants exhibiting the extremes of sculpturing and pilosity traits (images for pilosity are from AntWeb.org).

LabChart6 (V.6.1.3, 2008, ADinstruments Ltd., Dunedin, New Zealand). A pilot study found that the length of time a specimen was stored frozen (10 minutes or three months) did not affect outcomes.

**Hydrophobicity:** To determine the cuticular surface hydrophobicity (which declines with increasing wettability and adhesion energy), the molecular interactions between water droplets and the cuticular surface (including protuberances) were measured using methods modified from SUTER & al. (2004). Frozen ants from 26 species, 11 genera, and six subfamilies were tested (specimens were defrosted before measurements were taken). Between two and ten measurements were taken of a single body region per individual, depending on the relative condition and availability of specimens. A pilot study showed that length of time a specimen was stored frozen (10 minutes or three months) did not affect adhesion energy. In order to produce fine water droplets (< 0.3 mm to avoid the influence of gravity on droplet shape) on the cuticular surface, ants were held approximately 10 cm above an ultrasonic humidifier (Doulex USB donut-shaped humidifier, ABS, 5 × 5 × 2 cm, 40 g, Xiamen Pengchuang Trade Co., Ltd., Xiamen, China) resting in a glass beaker filled with distilled water.

To measure the contact angles of water droplets, digital images were taken and measured. Images that did not meet the following criteria were excluded: The diameter of the droplet was < 0.03 mm, the intersection between the water droplet and the surface was in focus, and the plane defined by the intersection between the water droplet and the surface and the geometric centre of the droplet was perpendicular to the camera's focal plane (Fig. S4). All procedures were carried out using a Leica M165C microscope and Leica software (Leica Application Suite 3.8.0) was used to measure contact angles and water droplet sizes.

The molecular-level interaction between water droplets and a surface when gravity is ignored is made up of the forces of cohesion between water molecules and adhesion between water molecules and the solid surface. If the cohesive forces are sufficiently larger than the adhesive

Tab. 3: Degrees of freedom (df), F-statistic and significance (* p = 0.05, ** p = 0.01, *** p = 0.001) from linear models testing the effect of the whole model including activity, location and subfamily on the first three principal components (detailed in Suppl. T1) (n = 59 species).

| Response | | PC1 | | | PC2 | | | PC3 | |
|---|---|---|---|---|---|---|---|---|---|
| R² | | 0.51 | | | 0.72 | | | 0.43 | |
| R²Adjusted | | 0.26 | | | 0.58 | | | 0.14 | |
| F | | 2.06 | | | 5.20 | | | 1.50 | |
| p | | 0.028 | | | <0.001 | | | 0.141 | |
| | df | F | p | | F | p | | F | p |
| activity | 1 | 0.02 | | | 16.33 | *** | | 0.44 | |
| location | 1 | 1.62 | | | 4.14 | * | | 0.01 | |
| subfamily | 6 | 6.36 | *** | | 11.97 | *** | | 3.10 | * |
| activity*location | 1 | 0.07 | | | 1.01 | | | 0.25 | |
| activity*subfamily | 4 | 0.06 | | | 2.53 | | | 1.36 | |
| location*subfamily | 4 | 1.18 | | | 1.48 | | | 0.68 | |
| activity*location*subfamily | 3 | 0.26 | | | 0.51 | | | 1.38 | |
| residuals | 38 | | | | | | | | |

Tab. 4: Number of replicate species (n), marginal and conditional R-square (R²m, R²c), degrees of freedom (df), Chi-square (χ²) and significance (* p = 0.05, ** p = 0.01, *** p = 0.001) from linear mixed models testing the effect of activity, pilosity, a cuticle measure (cuticle thickness or sculpturing), Weber's length and location on critical thermal maximum (CTmax), mass-independent water loss rate (MIWLR), puncture force and adhesion energy.

| Model | | $CT_{max}$ | $CT_{max}$ | MIWLR | | Puncture force | | Adhesion energy | |
|---|---|---|---|---|---|---|---|---|---|
| cuticle measure | | thickness | sculpturing | thickness | sculpturing | thickness | sculpturing | thickness | sculpturing |
| n | | 28 | 63 | 28 | 65 | 18 | 20 | 18 | 24 |
| $R^2_m$ | | 0.39 | 0.37 | 0.09 | 0.31 | 0.76 | 0.24 | 0.15 | 0.08 |
| $R^2_c$ | | 0.39 | 0.39 | 0.14 | 0.38 | 0.76 | 0.65 | 0.15 | 0.08 |
| Source | df | χ² p | χ² p | χ² p | χ² p | χ² p | χ² p | χ² p | χ² p |
| activity | 1 | 0.11 | 6.38 * | 2.44 | 2.13 | 0.10 | 0.65 | 0.09 | 0.02 |
| pilosity | 1 | 0.21 | 6.29 * | 0.02 | 4.46 * | 0.07 | 0.56 | 0.80 | 0.75 |
| cuticle measure | 1 | 1.13 | 8.34 ** | 0.05 | 5.40 * | 43.70 *** | 2.90 | 1.40 | 0.73 |
| Weber's length | 1 | 4.39 * | 6.58 * | 0.18 | 1.51 | 0.81 | 2.48 | 0.02 | 0.17 |
| location | 1 | 3.56 | 1.69 | 0.03 | 3.01 | 0.60 | 2.65 | 0.13 | 0.05 |

forces, the water droplet will be almost spherical (contact angle > 90°, Suter & al. 2004) and the surface is considered hydrophobic. Conversely if the forces of adhesion are larger than the forces of cohesion the droplet will tend to spread out on the surface, and the surface is considered hydrophilic (Young 1805, La-Place 1847).

The adhesion energy of water droplets was found using the relationship attributed to La-Place (1847) and Young (1805) as utilised by Suter & al. (2004).

$$W_a = \frac{W_c(1 + \cos\theta_c)}{2} = \gamma(1 + \cos\theta_c)$$

Where $W_a$ is the force of adhesion, $W_c$ is the force of cohesion, $\theta_c$ is the contact angle between the solid surface and the liquid, and $\gamma$ is the surface tension of the liquid. Therefore, by measuring the contact angle of the water droplet and the surface tension of the liquid (approximately 72 dynes / cm for distilled water at 25 °C), the adhesion energy was obtained, even for small cuticular protuberances.

**Data analysis:** Many cuticle traits are correlated, so to select key cuticle traits to use in analyses, correlations among all pilosity and sculpturing traits were tested.
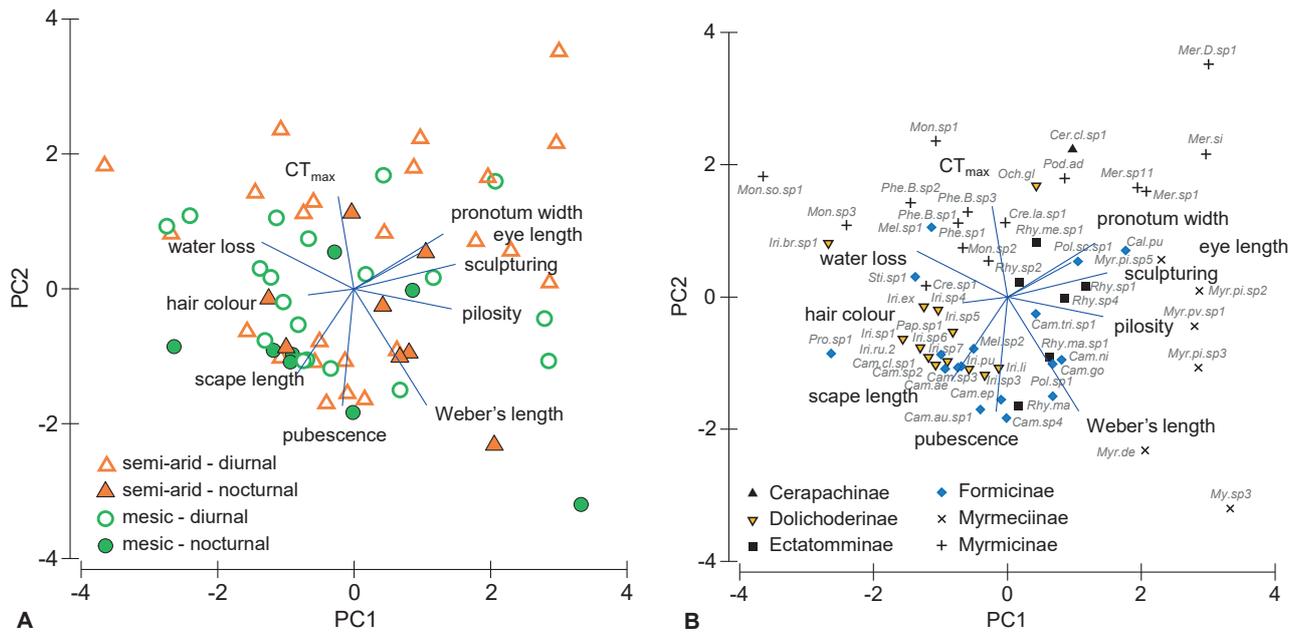
Fig. 2: Principal components plot, with ant species positioned on PC1 and PC2. Vectors indicate the strength of the relationship of traits with principal component axes. Species are coded by activity (diurnal / nocturnal) and location (semi-arid / mesic) in A) and by subfamily in B). Species name codes are provided in Table S2.

Where correlations were significant, a subset of variables were selected to use in further analyses.

To test how morphology differed among activity periods, locations and subfamilies, the eight morphological (including four cuticle traits) and two physiological traits (CT$_{max}$, mass-independent water loss rate) were reduced to three uncorrelated compound variables (PC1, PC2, PC3, Tab. S1) using principal components analysis (PCA) based on a correlation matrix in JMP (SAS INSTITUTE 2007). Data were normalised prior to analysis. Hardness (puncture force) and hydrophobicity (adhesion energy) were not used in this analysis as this data was not available for most species. General linear models on R were used to test the effect of the whole model including activity, location and subfamily on PC1, PC2 and PC3, independently.

Relationships among traits and response variables (CT$_{max}$, mass-independent water loss rate, puncture force and hydrophobicity) were analysed using general linear mixed models (GLMM) with a Gaussian distribution on R (R DEVELOPMENT CORE TEAM 2014). The effects of a model including the following predictors: activity (diurnal / nocturnal), pilosity, a cuticle measure (sculpturing or cuticle thickness), Weber's length and location were tested for each of the response variables individually. Subfamily was used as a random factor in all models to account for phylogenetic non-independence. Both marginal (fixed effects: R$^2_m$) and conditional (fixed + random effects: R$^2_c$) R$^2$ values (NAKAGAWA & SCHIELZETH 2013) were calculated using the package MuMIn (BARTON 2011). Weber's length (a measure of body size) was included for all analyses as body size is considered the most important physiological and ecological trait of animals (CALDER 1984, SCHMIDT-NIELSEN 1984, LOMOLINO & PERAULT 2007).

## Results

**Overview:** In total, 747 specimens (Scotia Sanctuary 378, La Trobe Wildlife Sanctuary 369) were collected from 70 species or morphospecies (Scotia Sanctuary: 40; La Trobe Wildlife Sanctuary: 30) in 17 genera (15; 14) in six subfamilies (6; 5). Approximately 17.5% and 33% of all ants collected from Scotia Sanctuary, and La Trobe Wildlife Sanctuary, respectively, were nocturnal. Both sculpturing and pilosity varied considerably among species (Table S2). Ants showed large variation in pilosity, having between zero and 127 hairs crossing the pronotum in profile. The ant with the greatest pilosity was in the genus *Myrmecia* FABRICIUS, 1804; *Calomyrmex purpureus* (MAYR, 1876) and *Iridomyrmex lividus* SHATTUCK, 1993 were also highly pilose. The least hairy ants were formicines in the genera *Stigmacros* FOREL, 1905 and *Polyrhachis* SMITH, 1857. Ants from the genera *Meranoplus* SMITH, 1853, *Myrmecia* and *Rhytidoponera* MAYR, 1862 had the highest sculpturing scores, whereas *Prolasius* FOREL, 1892, *Crematogaster* LUND, 1831 and *Monomorium* MAYR, 1855 were the least sculptured. Sculpturing was generally in the form of raised ridges forming tessellated cells (e.g. imbricate, areolate), which were almost always irregular hexagonal structures that differed in depth, wall thickness, area and frequency. Corrugations (or channels) (e.g., *Polyrhachis* and *Myrmecia* species) and simple excavations (e.g., *Rhytidoponera mayri* (EMERY, 1883) group sp. 1) were also found in several species.

**Correlations among cuticle traits:** Many of the measured traits indicating hairiness or cuticle structure were correlated (Fig. 1). Where correlations were significant, we selected a subset of variables to use in further
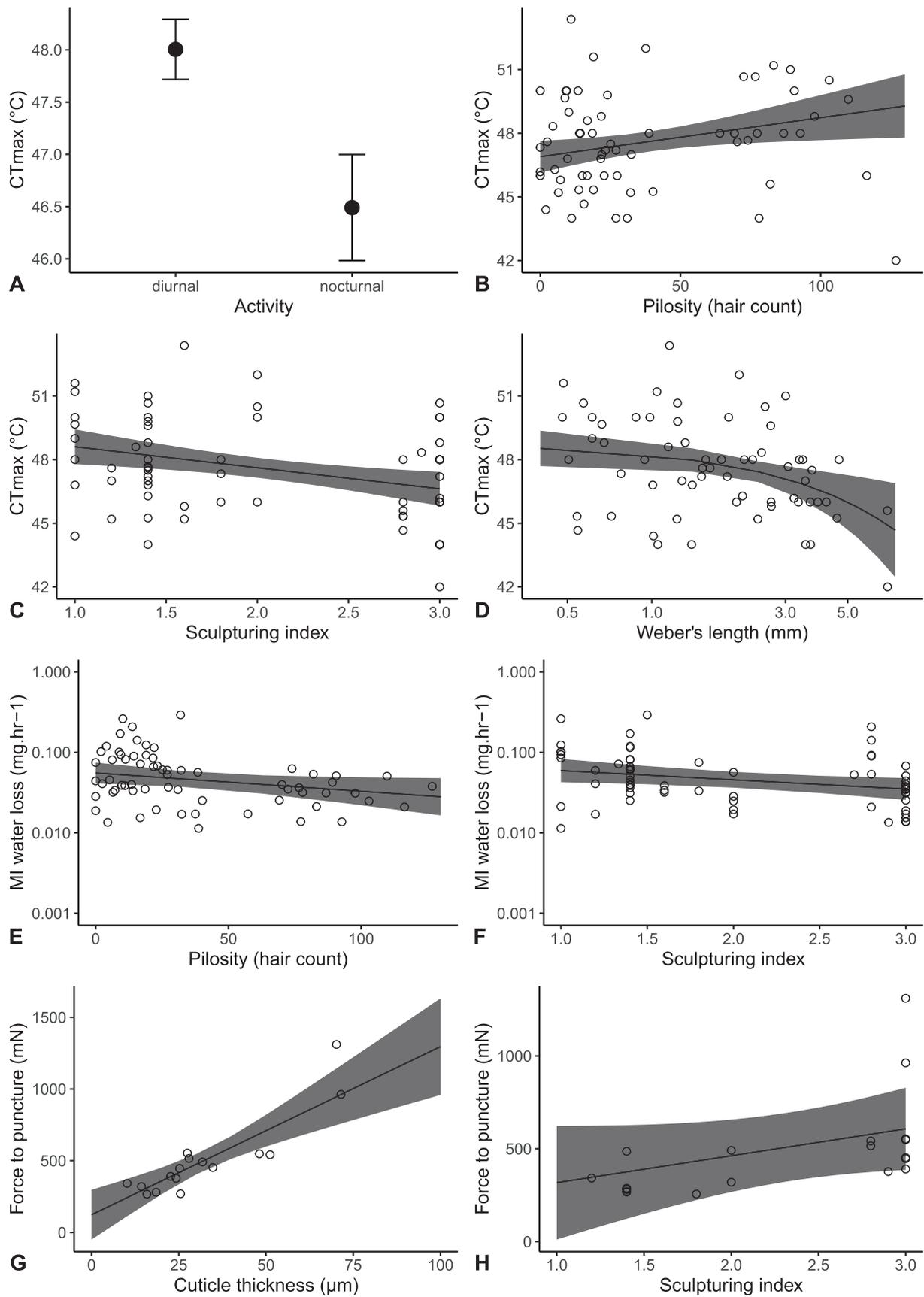
Fig. 3: Predicted relationships (and confidence intervals) among physiological and morphological or activity traits from linear mixed models: relationships between CTmax and A) activity; B) pilosity; C) sculpturing; and D) Weber's length; between mass-independent water loss and E) pilosity and F) sculpturing; and between puncture force and G) cuticle thickness and sculpturing.

analyses. For hairiness, we used "pilosity", "hair colour score" and "pubescence score". For cuticle structure, many variables were correlated, so we used only "sculpturing" and "cuticle thickness". Although sculpturing and cuticle thickness were correlated ($r = 0.64$, $p < 0.001$), we considered it valuable to establish how the more easily estimated "sculpturing" variable performed, compared with cuticle thickness.

**Axes of trait variation among species:** The first three axes of the principal component analysis (n = 59 species with complete data for all variables) explained 56.2% of variation in traits among species. PC1 was positively related to Weber's length, pronotum width (residuals), pilosity and pronotum sculpturing score and negatively related to mass-independent water loss (Tab. S1). PC2 was positively related to $CT_{max}$ and negatively related to scape length (residuals), Weber's length and pubescence score (Tab. S1). PC3 was positively related to mass-independent water loss rate and negatively related to pronotum width (residuals), hair colour score and $CT_{max}$.

**How do activity, location and subfamily affect trait complexes?:** Tests of the effect of the full model including activity, location and subfamily on each of the principal components revealed a key role for subfamily for all PCs and large variation in model fits (Adjusted R²: PC1 = 0.26; PC2 = 0.58; PC3 = 0.14) (Tab. 3). Diurnal ants were higher on PC2, indicating that they were smaller, with shorter scapes and had lower pubescence and higher temperature tolerance than nocturnal ants (Fig. 2A). The pattern was similar, but weaker, for location: ants from the semi-arid site were higher on PC2 than those from the mesic site. Myrmeciines clustered lower, while myrmicines were higher on PC2; formicines were low on PC1 (Fig. 2B).

**How well do pilosity and sculpturing predict ant physiology?:** Models that included cuticle thickness were generally weaker than those that included sculpturing, but this was probably because replication rates were lower for these models. For $CT_{max}$, both models had moderate predictive power (Tab. 4). In the model including cuticle thickness, $CT_{max}$ was predicted by Weber's length (negative), with location marginally non-significant (Tab. 4). When sculpturing was included, $CT_{max}$ was related to activity (higher for diurnal species), pilosity (positive), sculpturing (negative) and Weber's length (negative) (Fig. 3A - D).

For mass-independent water loss, the model that included cuticle thickness had weak predictive power, whereas that including sculpturing had moderate predictive power (Tab. 4). Mass-independent water loss was negatively related to both sculpturing and pilosity (Fig. 3E, F). Puncture force was strongly predicted by both models, but the model that included cuticle thickness was a stronger fit than the one including sculpturing (Tab. 4). Puncture force increased with cuticle thickness and sculpturing (marginally non-significant) (Fig. 3G, H). Adhesion energy was only very weakly predicted by our models (Tab. 4).

**Discussion**

A poor understanding of the functional importance of morphological variation among organisms is a major impediment to a predictive trait-based understanding of ecological communities. Using a cross-species approach, we addressed that knowledge gap for morphological traits of ant cuticles. First, we examined how ant cuticle traits relate to one-another. Next, we examined the key axes of trait variation for morphological and physiological traits and then asked how they related to subfamily, time of activity and location. Finally, we tested the functions of key cuticle traits. Cuticle traits were strongly interrelated and constituted part of an ecological strategy that encompassed multiple traits. The position of species along the key axes of trait variation differed among subfamilies, between diurnal and nocturnal ants, and between ants from semi-arid and mesic sites. We found support for a number of hypothesised functions of pilosity and sculpturing, including greater desiccation tolerance and physical defence, but relationships with $CT_{max}$ were more complex. We consider these findings and their importance for a predictive understanding of ecological communities.

**Correlations and axes of trait variation among species:** Several measures of cuticle structure were correlated, indicating that they were measuring similar features and suggesting that a single measure of cuticle structure would be sufficient to capture variation in this trait. Sculpturing was strongly correlated with cuticle thickness, indicating that the rapidly-obtained visual estimate "sculpturing" was a meaningful measure of the more time-consuming "cuticle thickness" and consistent with evidence that greater sclerotization is associated with denser cuticles (VINCENT & WEGST 2004). It took around 30 seconds to estimate "sculpturing" from a whole specimen in ethanol, but measurements of cuticle thickness included sectioning, drying and SEM set-up, measurement and coding, cumulatively took at least half an hour per specimen. There were fewer correlations among measures of cuticle hairiness: pilosity was correlated with the length of the longest hair but not hair colour score or pubescence, indicating that these traits are independent of one-another. Further, pilosity was weakly positively correlated with sculpturing, suggesting that more-pilose ants were more sculptured. We used these relationships to select variables to use in establishing the key axes of morphological and physiological variation in our ant assemblages.

Our ten morphological and physiological traits (including pilosity, sculpturing and the two uncorrelated hair traits) were reduced to three axes that explained much of the variation in ant traits. Several traits covaried, suggesting that they may be functionally or phylogenetically linked. In particular, species that had high pilosity also tended to have high sculpturing scores and relatively broader bodies, with greater desiccation tolerance. Larger ants tended to be more pubescent and this may have increased their thermal inertia, which may explain why their tolerance to high temperatures was greater. Higher

water content in larger ants might also have contributed to greater temperature tolerance. The inter-relatedness of these traits serves to highlight the difficulties of considering traits as independent properties of organisms, rather than as part of the complex of traits that makes up a species' ecological strategy (Westoby & al. 2002). We therefore considered the responses of ants in terms of their position along these trait axes.

**Do activity, location and subfamily predict the position of species along the key axes of trait variation?:** Unsurprisingly, phylogeny (subfamily) was a key determinant of the ecological traits of ants. This is consistent with previous studies that show a strong phylogenetic signal in the value of ant species traits (Gibb & al. 2015b, Blanchard & Moreau 2017). Myrmeciines were distinguished by their large body size and low $CT_{max}$, with the very largest species being nocturnally active. In contrast, myrmicines, which were largely active diurnally, were small, with a high $CT_{max}$. The phylogenetic links for these traits likely drove the unexpected negative relationship between $CT_{max}$ and body size, discussed in more detail below. Formicines were characterised by their long scapes, low pilosity and sculpturing and high rates of water loss, although *Calomyrmex purpureus* was a notable exception with its high sculpturing and pilosity. Many of the formicine species were nocturnal, crepuscular or active during the cooler parts of the day. However, the thermophilic genus *Melophorus* Lubbock, 1883, which is active in the hottest part of the day, tended to have a higher $CT_{max}$ than other formicines, with *Melophorus* sp. 1 having the highest $CT_{max}$ of all species examined. Dolichoderines (mainly *Iridomyrmex* Mayr, 1862) occupied a similar trait space to formicines, while ectatommines (mainly *Rhytidoponera*) were characterised by high sculpturing.

Nocturnally and diurnally active ants differed in their position along the axis (PC2) that represented body size and $CT_{max}$. Numerous previous studies have shown that $CT_{max}$ and body size are positively correlated in ants and other insects (Clémencet & al. 2010, Oberg & al. 2012, Ribeiro & al. 2012, Kaspari & al. 2015), that is, that increasing body size increases a species' ability to withstand higher temperatures. This contrasts strongly with our finding that $CT_{max}$ and body size increase in opposite directions along PC2, although a similar pattern has been observed in some ant assemblages (Verble-Pearson & al. 2015, Nowrouzi & al. 2018). This anomaly may be explained by the link between these traits and time of activity: Nocturnal species experience much lower maximum temperatures than diurnal species (Esch & al. 2017) and tended to be larger in our study. The relationship between body size and time of activity observed within single sites in this study reflects the tendency of ectotherm body size to increase in cooler climates toward the poles (Cushman & al. 1993, Chown & Gaston 2010, Gibb & al. 2018) and the tops of mountains (Bishop & al. 2016). A raft of explanations for this relationship have been suggested, including starvation resistance in seasonal environments (Heinze & al. 2003), greater oxygen availability in cold climates

(Atkinson 1996) and maturation at smaller sizes due to the positive effect of high temperatures on metabolic rates (Gillooly & al. 2001). For this study, the last explanation is unlikely, given that most ants mature in a temperature-buffered underground environment. It is unclear whether there are meaningful differences in seasonal availability of resources or oxygen between day and night.

Species from semi-arid sites showed similar adaptations to diurnal ants, probably largely reflecting the difference in temperature tolerance ($CT_{max}$) that was linked to body size and phylogeny. Variation within sites was large, in agreement with previous studies that have shown that up to 80% of global morphospace (T.R. Bishop, unpubl.) and 74% of global variation in $CT_{max}$ (Kaspari & al. 2015) can be maintained within single local assemblages. This is probably a result of high variation in local microhabitats and microclimates, both spatially and temporally, and the ability of species to select microclimates behaviourally. In this study, differences between mesic and semi-arid environments may also reflect our efforts to sample across the spectrum of species, independent of their abundance. While species with a diverse array of trait values may be present at most sites, only a few of those species will be abundant. Relative abundance (rather than presence) is more likely to reflect the differences in ecological conditions.

**Does pilosity predict ant physiology?:** Previous studies have revealed a role for pilosity in functions including defence against predation, hydrophobicity, mechanoreception, desiccation-resistance and thermoregulation (Tab. 1). We showed that pilosity is important in predicting both the $CT_{max}$ and water loss rate across species, but not adhesion energy or puncture force. Although pilosity tended to increase with body size in our study, body size was accounted for in our analyses, so was not the driver of these patterns. $CT_{max}$ (heat tolerance) increased with increasing pilosity, supporting a thermoregulatory function for pilosity. This is consistent with evidence from plants that leaf trichomes modulate leaf temperature by reflecting light (Ehleringer & al. 1991) and that reflection of near infra-red wavelengths by hairs can influence body temperatures (Shi & al. 2015, Stuart-Fox & al. 2017). However, $CT_{max}$ was measured in thermal baths in the lab, so ants were not exposed to sunlight and it is more likely that insulative properties of pilosity came into play. Hairs may protect a warm boundary layer from disturbance, allowing absorbed heat to be retained by reducing convective heat exchange (Casey & Hegel 1981, Kevan & al. 1982), perhaps explaining why the pilosity of spiders increases with declining environmental temperature (Gibb & al. 2015a). Increased insulation through increased pilosity may also allow cool air in the boundary layer to be retained, thus increasing $CT_{max}$.

Mass-independent water loss rate declined with increasing pilosity, suggesting that hairs reduce water loss. This is consistent with evidence that hairs aid in desiccation resistance in caterpillars (Cloudsley-Thompson 1958, Casey & Hegel 1981, Kevan & al. 1982). Although

the pilosity of ants never approaches that of invertebrates such as woolly caterpillars (Casey & Hegel 1981, Fields & McNeil 1988), it may be that even small increases in pilosity increase desiccation tolerance.

We were unable to detect any relationship between pilosity (or sculpturing) and adhesion energy, suggesting that these traits do not affect the hydrophobicity of ant cuticles. This is in contrast to woolly bear caterpillars, in which hairier individuals were less likely to drown (Meyer-Rochow 2016), and plants, where increasing density of leaf trichomes decreases leaf wettability and droplet retention (Fernández & al. 2017). If hydrophobic properties are better measured at finer visual resolution, for example, using SEM (Bush & al. 2008), then our measures of pilosity and sculpturing are unlikely to be important in the ability of ants to deal with dew, rain or other water in their environment.

**Does sculpturing predict ant physiology?:** Sculpturing was important in predicting $CT_{max}$, water loss rate and puncture force, but not adhesion energy. Models including sculpturing better predicted $CT_{max}$ and water loss rate than those that included cuticle thickness. However, this is probably largely because we were able to obtain sculpturing measures for at least twice as many species, making models more powerful.

No previous studies have investigated the relationship between $CT_{max}$ and cuticle sculpturing, but reduced cuticular thickness is associated with increased thermal conductance and reduced heat tolerance in insects (Galushko & al. 2005, Amore & al. 2017). We therefore predicted that more sculptured or thicker cuticles would provide greater protection against high temperatures. Surprisingly, cuticle sculpturing and $CT_{max}$ were negatively related. This might be because thicker or more sclerotised cuticles contain less water, which may influence the ability of ants to cope with heat stress. However, many of the ant species with higher sculpturing scores (such as *Myrmecia* and *Rhytidoponera* species) were larger ants that were active at cooler temperatures at night or early in the morning. Body size is also correlated with a range of physiological factors that are linked with $CT_{max}$, including variation in heat-shock protein synthesis (Gehring & Wehner 1995, Nguyen & al. 2016, Stanton-Geddes & al. 2016, Willot & al. 2017), cuticular thickness (Galushko & al. 2005), cuticular lipid contents (Hood & Tschinkel 1990). A larger dataset may be necessary to disentangle the effects of nocturnality and body size on both $CT_{max}$ and sculpturing.

Increased cuticle sculpturing was associated with lower mass independent water loss rates, although this relationship was not particularly strong ($R^2m = 0.31$). The hydrophobic lipid layer and ventilation pattern are usually considered most critical in the water relations of insect cuticle (Toolson & Hadley 1977, Lighton & Feener 1989), so it is surprising that our simple measure of sculpturing was indicative of water loss rate. Although increased sculpturing is probably linked with a greater surface area to volume ratio (which should increase water loss), it is also linked with cuticle thickness and possibly unmeasured traits such as cuticle porosity, which might influence water loss. Further, sculpturing may be related to the nature of cuticular lipids or melanin in the cuticle, both of which could affect water loss (Gibbs & Rajpurohit 2010, Matute & Harris 2013, King & Sinclair 2015, Sprenger & Menzel 2020).

In contrast to $CT_{max}$ and water loss rate, "puncture force", a key mechanical property, was best predicted by the model including cuticle thickness. In this case, similar numbers of species were measured for sculpturing and cuticle thickness, but model fit was three times better for cuticle thickness. This is consistent with previous findings that cuticle thickness is an indicator of insect "hardness" (Evans & Sanson 2005). Subfamily was used as a random effect in our models, so its effect is unclear. However, previous work suggests that there is also a strong phylogenetic signal to cuticle structure, with dolichoderines and formicines having thin cuticles that readily fracture when dry, whereas the cuticles of myrmicines, myrmeciines, ponerines, ectatommines are thicker and retain their hardness and resistance to fracture even when dry (Wheeler 1910). Thicker cuticles in ants might provide structural support or may play a defensive role. In support of a protective role, isotope analyses show that more predatory ants have more structured cuticles (Gibb & al. 2015b). Further, a higher degree of sculpturing is found in structurally important areas (areas that suffer greater strain from attacks or general wear and tear), such as the edge of the pronotum and the neck joint (Hunt 1983, Hölldobler & Wilson 1990).

**Conclusions:** The study of traits has increased greatly in recent years, with a renewed focus on traits as an approach to better understand the vast diversity of invertebrates and their ecology (Moretti & al. 2017, Brousseau & al. 2018, Wong & al. 2019). In this study, we took a mechanistic approach to understanding the physiological function of two easily measured cuticle traits (sculpturing and pilosity) that are receiving increasing attention in trait-based studies of ant ecology (Gibb & al. 2015b, Parr & al. 2017, McElroy & Donoso 2019). We showed links between these traits and temperature and desiccation tolerance, as well as cuticle strength. It might be argued that direct physiological measures should be used in preference to these indicators. However, the speed with which meaningful trait measures can be obtained across a broad range of species through preserved specimens and online images such as those held on AntWeb (www.antweb.org) means that it is achievable to obtain data at a rate that will allow us to address questions at a global scale.

While difficulties remain in how we should deal with the non-independence of traits and their contribution to a whole-of-organism ecological strategy (Westoby & al. 2002), it remains vital that we use mechanistic studies to establish the function of specific traits. Where these are difficult to measure, the use of correlated indicator traits, such as those tested here, may prove fruitful. Developing a solid cohesive foundation for trait-based ecology will take a colossal effort over many years. However, the po-

tential of the trait-based approach to ecology to produce accurate predictive models of assemblage responses to global change, as well as to provide general rules for ecology, certainly make this approach worth the considerable time and effort.

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## Author contributions

JB, KR and HG conceived the ideas; JB, KR, TD and AM designed methodology; JB collected the data; JB and HG analysed the data; JB and HG wrote the manuscript; KR, TD and AM contributed to manuscript drafts.

## Data accessibility

All data used in this manuscript are available in Table S2, as digital supplementary material to this article, at the journal's web pages.

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