

Evolution of cheaper workers in ants: a comparative study of exoskeleton thickness

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The great evolutionary success of insects is partly linked to the innovation of the cuticle, which underlies a complex exoskeleton with varied functions. Cuticle development is costly because chitin and cuticular proteins require ingested nitrogen. Studying cuticle thickness on a comparative basis allows insight into the trade-off between offspring quality and quantity. This trade-off takes a particular meaning in ant workers because they are wingless, non-dispersers and generally sterile. We selected a comprehensive sample of 42 ant species (40 genera belonging to nine subfamilies) and measured cuticle thickness in workers using labour-intensive histological sectioning of the prothorax. Thickness varied substantially across genera, ranging from 1.3 to 109.8 μm . We correlated thickness with body size, measured as head width. Our results show a strong influence of body size, but also phylogeny, because workers in ‘formicoid’ species tend to have a thinner cuticle (two exceptions). The effect of body size is also obvious in species having size-polymorphic workers. We discuss the idea that a thinner cuticle is associated with miniaturization of workers, and its ecological consequences are buffered in a social environment. We reveal that the bulk of ant species have a very thin cuticle, and this represents cost savings that may partly underlie the massive increase in colony size during adaptive radiation.

ADDITIONAL KEYWORDS: allocation tradeoffs – body size – chitin – colony size – cuticle – nitrogen limitation.

INTRODUCTION

The cuticular exoskeleton of arthropods is a key adaptation underlying their exceptional evolutionary success. Cuticle functions both as a barrier against desiccation and pathogens, as well as a skeleton (for mechanical protection and muscle attachment) (Vincent & Wegst, 2004). Insect cuticle consists of chitin nanofibres embedded in a matrix of proteins, polyphenols and water (Vincent & Wegst, 2004; Zhu *et al.*, 2016). Its development has a substantial cost because ingested nitrogen is required for chitin and proteins. Resources allocated to the exoskeleton are determined by body size but also cuticle thickness, although variability in the latter has not been investigated across insects. In ants, we do not know if cuticle thickness is uniform across lineages (the single data point is *Cephalotes atratus*

with gaster cuticle thickness of $19.2 \pm 0.65 \mu\text{m}$; Verble *et al.*, 2012).

Ant colonies produce two types of female offspring: queens and workers. Workers are wingless and generally lack direct fitness since they help queens to reproduce. Although they lack dispersal and reproductive constraints, ant workers are capable of considerable physical feats (e.g. forage over long distances, predation, defence and carry heavy objects), and this holds across a great diversity of habitats ranging from arid to very humid. Colony size varies from 10 to 10^6 adults according to clades, and it is unknown whether an increased investment in worker number corresponds to lower individual quality. Body size (often used as a proxy for offspring quality) of ant workers varies from 1 mm to over 25 mm across species. The trade-off between quality and quantity in sterile offspring has received little attention (Molet, Van Baalen & Peeters, 2008), and body size is evidently not the only measure of quality.

Miniaturization in insects is based on a reduction of internal structures and the exoskeleton (Polilov,

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2015). The body size of ant workers decreased sharply during ant evolution (Peeters & Ito 2015), and miniaturized workers are particularly widespread in formicoid subfamilies (a monophyletic clade comprising 90% of extant ants; Ward, 2014). Thus, the effects of phylogeny and body size on cuticle thickness need to be untangled across the ants. A subjective impression based on dissection of internal organs is that the exoskeleton in formicoid subfamilies is thinner and more flexible than in poneroid subfamilies. Billen (2009) documented the widespread occurrence of intersegmental glands with a lubrication function in the subfamily Ponerinae, unlike in formicoid subfamilies. This is indirect evidence of a thick cuticle requiring lubrication to ease movement between segments. Using histological sectioning of the prothorax, we compared cuticle thickness of workers among 17 poneroid species (subfamilies Amblyoponinae, Leptanillinae, Paraponerinae and Ponerinae) and 25 formicoid species (subfamilies Dorylinae, Dolichoderinae, Formicinae, Myrmicinae and Pseudomyrmecinae). We analysed whether poneroids have a thicker cuticle than formicoids (controlled for body size), and we quantified the link between body size and cuticle thickness across species, subfamilies and phylogenetic clades (poneroid vs. formicoid). The intraspecific effect of body size was also investigated in several formicoids having size-polymorphic workers. We discuss the hypothesis that an evolutionary decrease in cuticle thickness permits workers that are cheaper to manufacture, thus allowing increases in colony size whenever adaptive.

MATERIAL AND METHODS

SPECIES SAMPLING

Ants exhibit tremendous inter-generic variability in worker body size, and we aimed to sample this variability across nine different subfamilies (Appendix 1): Amblyoponinae (2 species), Paraponerinae (1), Ponerinae (13), Leptanillinae (1), Pseudomyrmecinae (1), Dorylinae (2), Formicinae (6), Dolichoderinae (4) and Myrmicinae (12). Head width was used as a proxy of body size. We also included eight genera with highly size-polymorphic workers or soldiers (coefficient of variation for head size >10): *Atta cephalotes*, *Carebara* (formerly *Pheidologeton*) *diversa*, *Cataglyphis bombycina*, *Dorylus orientalis*, *Messor minor*, *Myrmica rogeri*, *Pheidole noda*, *Solenopsis geminata* and *S. invicta*. We restricted our interspecific comparisons to worker castes, which are the principal component of colonies. Two social wasps (*Parachartergus smithii*, *Protopolybia amarella*) were included for comparison.

HISTOLOGY

Keller, Peeters & Beldade (2014) showed that neck muscles are highly developed in all ant workers. Some of these powerful muscles are attached to the pronotum (dorsal plate of the first segment), and hence strong constraints exist on the rigidity of this part of the exoskeleton, and this is what we chose to measure.

Using microsurgery scissors, the thorax was cut transversely between the fore- and midlegs to allow proper penetration of the chemicals used for tissue processing. The prothorax with the head attached was fixed in 2% cold glutaraldehyde in a 50 mM Na-cacodylate buffer at pH 7.3 containing 150 mM sucrose. After post-fixation in 2% osmium tetroxide in the same buffer, it was dehydrated in a graded acetone series. Just before embedding, the head was detached from the prothorax in order to measure head width. The prothorax was embedded in Araldite. Serial transverse sections with a thickness of 1 µm were made with a Leica EM UC6 ultramicrotome, stained with methylene blue and thionin and viewed with an Olympus BX-51 microscope.

Preliminary comparisons of longitudinal and transverse sections showed that the thickness of pronotum cuticle is constant in all directions (Fig. 1). To standardize, we consistently made transverse sections in the region where the pronotum reaches its maximal width (Fig. 1). Cuticle thickness was measured on digital section micrographs using the Olympus DP-Soft version 3.2 program.

DATA ANALYSIS

Statistical analyses were performed with R 2.13 (available at <http://cran.r-project.org/>). To assess the relationships between cuticle thickness and body size or phylogeny, we used linear mixed-effects models (lme procedure). We modelled 'cuticle thickness' as a function of the two fixed variables 'head width' and 'clade' (poneroids vs. formicoids) and their interaction, and the two random variables 'subfamily' and 'species' ('species' being nested within 'subfamily'). Models including or excluding one of the variables were compared using the ANOVA procedure to test the effect of this variable. 'Cuticle thickness' and 'head width' were log-transformed to meet the assumption of normality, and residual homoscedasticity was verified. Because the variance of 'clade' residuals was heterogeneous, we allowed for heteroscedasticity in our model using the weights procedure.

We also computed the ratio between cuticle thickness and head width for each individual to think about differences between clades and species (Appendix 2).

To describe the rate at which cuticle thickness changes with head width depending on clade, subfamily or species, we log-transformed 'cuticle thickness' and 'head width'

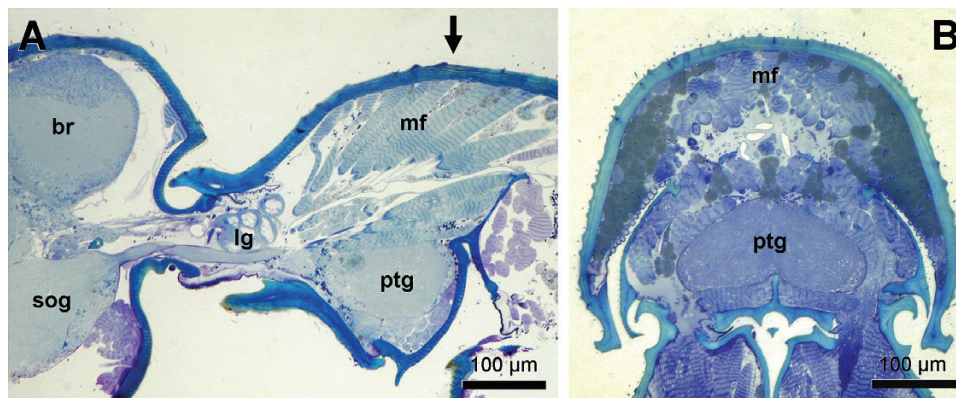


Figure 1. A, longitudinal section through prothorax of *Proceratium japonicum*, showing the plane of cross-sectioning (arrow); B, cross-section where thickness was measured (br: brain, lg: labial gland, mf: muscle fibres, ptg: prothoracic ganglion, sog: suboesophageal ganglion).

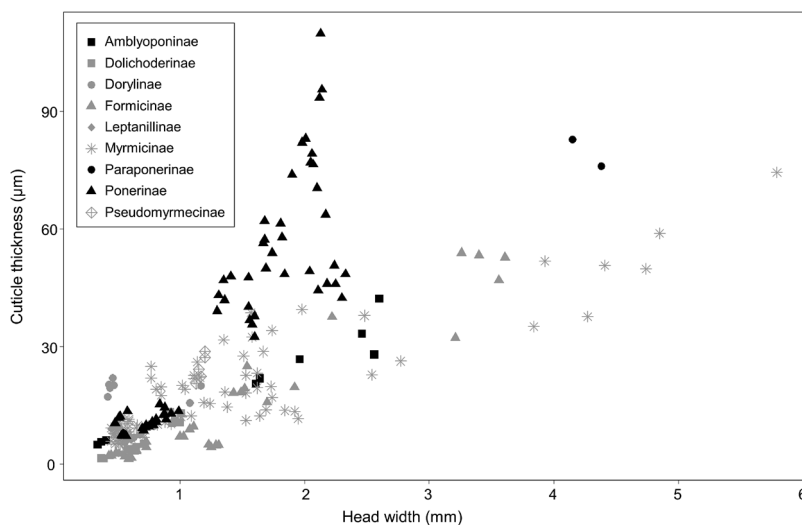


Figure 2. Allometry between cuticle thickness and head width across nine ant subfamilies. Poneroid (black) and formicoid (grey) subfamilies are shown.

and performed standardized major axis regressions (sma procedure in R). The allometry coefficients, i.e. slopes of the regression lines, were extracted and compared between clades. When the allometry coefficient equals 1, cuticle thickness and head width grow at the same rate (isometry). When it differs from 1, cuticle thickness and head width grow at a different rate (allometry).

RESULTS

Cuticle thickness was significantly and positively correlated with 'head width' but not with 'clade' (respectively L-ratio = 350.72, $P < 0.0001$; L-ratio = 1.94, $P = 0.16$), with no interaction between these two variables (L-ratio = 0.0001, $P = 0.99$). Thickness was also affected by the nested random variables 'species' and

'subfamily' (L-ratio = 350.72, $P < 0.0001$ for the two variables; L-ratio = 10.08, $P = 0.002$ for subfamily only). This showed that differences in cuticle thickness among subfamilies and among species were large and accounted for most variations between the poneroid and formicoid clades (Fig. 2). Variance in cuticle thickness was 3.2 times higher in formicoids than in poneroids (L-ratio = 25.55, $P < 0.0001$).

Among the 42 species studied, 15 species with the lowest cuticle/head ratio were all formicoids, whereas five of the 15 species with the highest ratio were formicoids (Fig. 3). Some poneroids such as *Odontoponera* and *Diacamma* have an extremely thick cuticle, but so do some formicoids such as *Ooceraea* and *Tetramorium* (ratio medians 0.039, 0.044, 0.044 and 0.024, respectively). The formicoid *Anoplolepis* had the thinnest cuticle, whereas the thinnest poneroid *Cryptopone* was much

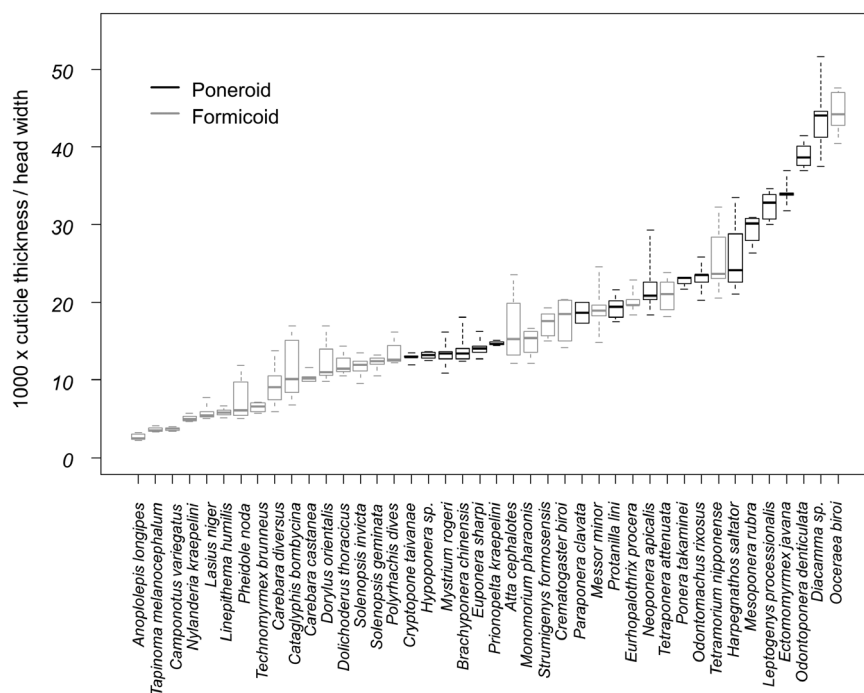


Figure 3. Ratio between cuticle thickness (prothorax) and head width in poneroids (black) and formicoids (grey). Medians, quartiles and ranges are shown.

thicker (ratio medians 0.002 and 0.013, respectively). Examples representative of the entire ranges of body size in poneroids and formicoids are shown in Figure 4.

We assessed the allometric growth rule between cuticle thickness and head width (Fig. 2). When pooling all individuals from all species and subfamilies, cuticle thickness increased more rapidly than head width in both poneroids and formicoids (correlation $R^2 = 0.84$ $P < 10^{-6}$ and $R^2 = 0.57$ $P < 10^{-6}$, respectively; allometry coefficients 1.45 and 1.50, respectively; tests against 1: $P < 10^{-6}$), and there was no difference in allometry between the two clades ($P = 0.61$). For a similar head width, cuticle thickness was higher in poneroids than in formicoids (elevations 1.29 and 1.05, respectively, $P < 10^{-6}$). Allometries differed among subfamilies (Table 1). Focusing on intraspecific effects, we selected species with workers that are highly variable in body size (coefficient of variation for head width >10) (Table 2). We found that cuticle thickness increased at the same rate, or more quickly, or more slowly than head width or not at all, depending on species, but it never decreased. The range of cuticle thickness among size-polymorphic workers is illustrated for *Dorylus orientalis* (Fig. 5).

DISCUSSION

INTERACTION BETWEEN BODY SIZE AND PHYLOGENY

Our comparative survey of all major ant lineages revealed striking variation in exoskeleton thickness of

workers (Fig. 4), ranging from 1.3–1.8 μm (*Anoplolepis* with head width 0.56–0.61 mm) to 76.9–109.8 μm (*Diacamma* with head width 2.01–2.14 mm). Analyses based on mixed effects models showed that body size (as measured by head width) is strongly correlated with cuticle thickness. Species at the lower end of the range of body sizes have an extremely thin cuticle. However, this relationship differs between poneroid and formicoid clades; that is, at comparable body sizes, poneroids have thicker cuticle than formicoids. The great variations within each clade signal that appropriate comparisons can only be done at the level of subfamilies. And even so, variations within the highly speciose subfamily Formicinae are especially high. Environmental conditions (aridity or high humidity) may account for some of this variability. Canopy ants show high levels of resistance to desiccation (Bujan, Yanoviak & Kaspari, 2016), and future studies need to assess the ecological and life-history consequences of cuticle thickness.

Comparative data suggest strongly that the generally thicker cuticle of Ponerinae is a basal trait, although this involves assumptions about the body size of the first ants. Our data for two social wasps [*Parachartergus smithii* (40.8 μm , head width 3.4 mm) and *Protopolybia amarella* (14.7 μm , head width 1.6 mm)] show an even thicker cuticle than the thickest Ponerinae, i.e. *Odontoponera* and *Diacamma* (cuticle/head ratios = 0.012 and 0.009 vs. 0.039 and 0.044, respectively). Although wasps have flight constraints, their thick cuticle supports the notion that the first

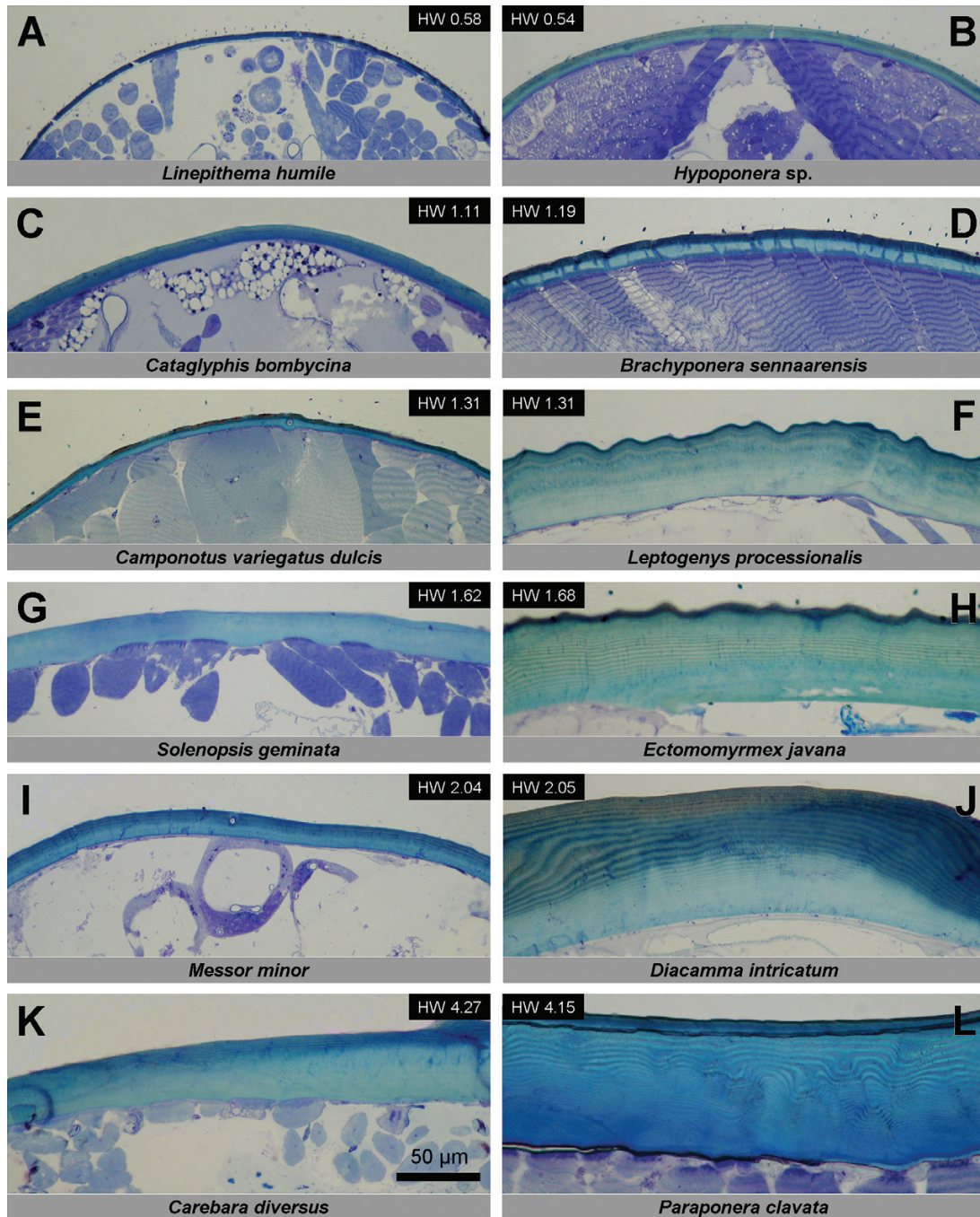


Figure 4. Cross-sections through pronotal cuticle in various species (formicoids on left, poneroids on right), comparing workers of similar head widths in each row (AB, CD, etc.). Head width (HW, in millimetres) increases from upper to lower rows. All photographs at same magnification.

ants had a thick cuticle also. Within the Ponerinae, genera unrelated to each other show a reduction in cuticle thickness, such as *Anochetus graeffei* (data not shown), *Cryptopone taivanae* and *Hypoponera* sp. These genera are characterized by minute workers and colonies can be more populous, a similar trend as found in formicoids.

WORKER SIZE POLYMORPHISM AFFECTS CUTICLE THICKNESS

Our data on intraspecific variability in cuticle thickness are novel and show clearly how body size affects cuticle thickness. When body size increases, cuticle thickness increases much more quickly ('cuticle thickness' is

Table 1. Multiple comparisons of allometry coefficients among subfamilies; no significant correlation was detected between head width and cuticle thickness in Dorylinae and Leptanillinae, so these are not shown here

Clade	Subfamily	Allometry coefficient	Ambly.	Pone.	Doli.	Formi.	Myrmi.	Pseudo.
Poneroids	Amblyoponinae	0.95	–	***	***	***		
	Ponerinae	1.71	***	–	***		***	
Formicoids	Dolichoderinae	2.26	***	***	–		***	
	Formicinae	1.81	***			–	***	
	Myrmicinae	1.11		***	***	***	–	
	Pseudomyrmecinae	5.18						–

***Significant difference between two allometry coefficients ($P < 0.001$).

Table 2. Intraspecific allometry between head width and cuticle thickness; only species with highly size-polymorphic workers or soldiers (coefficients of variation for head size >10) are included

Species	Correlation			Allometry coefficient	Test against isometry	Conclusion
	<i>N</i>	<i>R</i> ²	<i>P</i>		<i>P</i>	
<i>Atta cephalotes</i>	9	0.87	$< 10^{-3}$	0.69	0.026	Allometry < 1
<i>Carebara diversa</i>	14	0.91	$< 10^{-6}$	0.99	0.94	Isometry
<i>Cataglyphis bombycina</i>	12	0.96	$< 10^{-6}$	1.57	$< 10^{-4}$	Allometry > 1
<i>Crematogaster biroi</i>	6	0.07	0.61	NA	NA	
<i>Dorylus orientalis</i>	9	1	$< 10^{-6}$	1.68	$< 10^{-6}$	Allometry > 1
<i>Messor minor</i>	5	0.47	0.2	NA	NA	
<i>Mystrium rogeri</i>	6	0.78	0.02	1.21	0.46	Isometry
<i>Pheidole noda</i>	9	0.97	$< 10^{-5}$	1.54	$< 10^{-3}$	Allometry > 1
<i>Solenopsis geminata</i>	7	0.97	$< 10^{-4}$	0.91	0.3	Isometry
<i>Solenopsis invicta</i>	9	0.9	$< 10^{-4}$	1.23	0.12	Isometry

proportional to 'head width' to the power 1.5) (Table 2). Ten species with highly polymorphic workers (including soldiers) exhibited great variability in cuticle thickness, mostly consistent with head width. In *Carebara* (formerly *Pheidologeton*) *diversa*, minute workers (head width 0.55–0.61 mm) with a cuticle 5.4–8.1 µm coexist with soldiers (head width 4.27–4.74 mm) having a cuticle 37.6–50.6 µm. All size intermediates exist also, but the majority of colony members are small with a thin exoskeleton. In several formicoid species, size-polymorphic workers represent adaptations for cost-efficient performance of specialized tasks (Powell, 2008). Both major workers and soldiers (Molet, Maicher & Peeters, 2014) are more likely to be involved in defence, where a thicker cuticle may bring benefits.

DOES EXOSKELETON THICKNESS AFFECT SURVIVAL?

Cuticle is a versatile composite material and its multiple functions are difficult to untangle: mechanical strength and muscle attachment; barrier to disease (fungal pathogens) and desiccation; and abrasion resistance (Zhu *et al.*, 2016). All body parts of an insect

are made from cuticle, but they may have different physical characteristics (e.g. legs and mandibles; Dirks, Parle & Taylor, 2013). Cuticle thickness is likely to vary among body parts and this needs investigation across ants. Furthermore, the biomechanical properties (e.g. puncture resistance, tensile strength, peak strain) of thin vs. thick cuticles are unknown for ant workers.

What are the penalties of a thinner exoskeleton in social insects? Because age polyethism universally underlies the division of labour, it is only the older adults that forage outside the nest. A thinner cuticle may expose workers to greater danger in fighting or desiccation, but these drawbacks come under selection only at the end of workers' lives. Moreover, foragers often suffer high mortality due to predation, and hence they are exposed to outside risks for only days or weeks (Schmid-Hempel & Schmid-Hempel, 1984; Kwapich & Tschinkel, 2016), and durability of body parts (Dirks *et al.*, 2013) may not be an issue.

A perennial nest (i.e. protection of younger individuals) combined with age polyethism removes many constraints on the requirements of an exoskeleton. Young workers are not handicapped by thin cuticle – indeed,

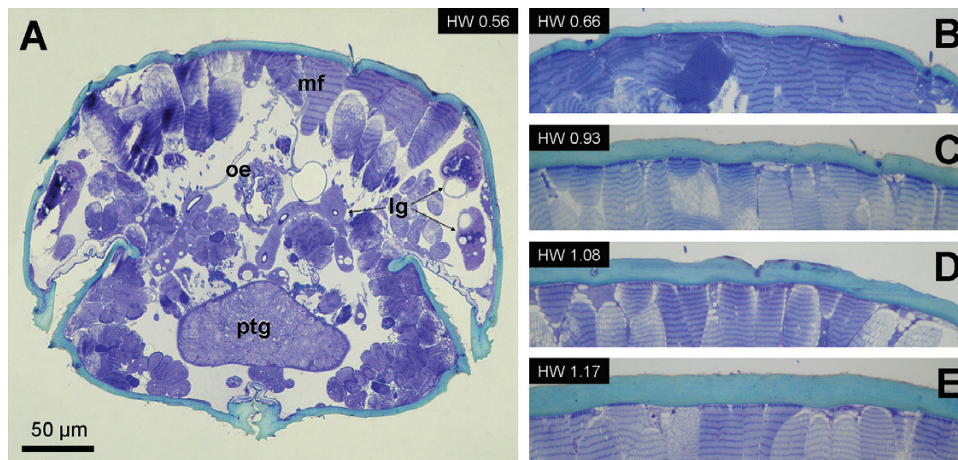


Figure 5. Cross-sections through prothorax of smallest worker (head width 0.56 mm) of *Dorylus orientalis* (A) and of larger conspecific workers (B–E) with indication of head width (HW). Note increase of cuticle thickness with increasing worker size. All photographs at same magnification (oe: oesophagus, lg: labial gland, mf: muscle fibres, ptg: prothoracic ganglion).

they metamorphose into adults with an incompletely sclerotized cuticle, suggesting that desiccation or physical protection is not a problem. A decrease in cuticle thickness is apparently buffered by the social environment, and hence producing workers with a more cheaply manufactured exoskeleton may be selected in a social context. Similarly in termites, cooperative behaviours mitigate the cost of a fragile morphotype and cuticle can be thin (Nalepa, 2011).

CHEAPER EXOSKELETON ALLOWS LARGER COLONIES

Comparative data on cuticle thickness in insects are scant, and there exist no estimates of how it affects manufacturing costs of the exoskeleton. In a cockroach, the shed exuvium at moulting is 15.9% of the dry weight in young instars, and 8.9% in older juvenile stages, which amounts to a large portion of the nitrogen budget (from Nalepa, 2011). Cost savings from a thinner cuticle may be minuscule for one individual, but savings are considerably amplified in the populous colonies of social insects. It is likely that the exoskeleton represents a substantial component of colonial expenditure. Given that life-history trade-offs are due to resource limitation, central place foraging ant colonies have finite food resources that affect either per-worker allocation or the number of worker offspring.

As opposed to total cost of manufacture, ecological stoichiometry gives a different perspective on cuticle thinning. Cuticle requires nitrogen for chitin and proteins. In termites, Nalepa (2011) pointed out that nitrogen not locked up in the cuticle can become available for the production of additional colony members. Ponerine workers of many species have heavy, melanized cuticle. This expenditure seems possible because

of a carnivorous diet, which represents high nitrogen. Many formicoid species (especially Formicinae and Dolichoderinae) shifted from strict carnivory to foraging for honeydew or other sweet exudates, and hence nitrogen content becomes a constraint (Davidson, 2005). Do formicoid workers get rid of a thick cuticle to reduce nitrogen expenditure? Although it is tempting to hypothesize a link between the scarcity of nitrogen in plant exudates and reduced cuticle, this needs investigation and explanation of causality. Besides nitrogen, other potentially limiting elements (e.g. zinc and manganese) are needed for the development of ant cuticle (Schofield *et al.*, 2003). Yet another perspective is the benefit provided by microbial symbionts living inside the larvae of camponotine and Cardiocondyla ants: Blochmannia and other bacteria synthesise aromatic amino acids essential for cuticle sclerotization of young adults (Russell *et al.*, 2017).

Assuming that the species we investigated are representative of entire genera, species with thinner cuticle (the lowest ratios of cuticle thickness/head width in Fig. 3) give information about some of the most speciose ant genera, i.e. *Camponotus* (1030 species), *Pheidole* (1009), *Carebara* (204), *Solenopsis* (197), *Dolichoderus* (132), *Lasius* (114), *Nylanderia* (109), *Technomyrmex* (93) and *Dorylus* (60) (www.antcat.org). Indeed, given that the subfamilies Myrmicinae, Formicinae and Dolichoderinae comprise about 75% of all ant species, our data suggest that the bulk of ant workers have a thin cuticle. Nonetheless, formicoid genera with thicker cuticle are also successful, such as *Tetramorium* (568 species), *Crematogaster* (508) or *Polyrhachis* (709). A thicker armoured exoskeleton may be adaptive during aggressive interactions with prey or competing ants, but this needs investigation.

Winglessness of ant workers allowed a drastic reduction in body size, but also simplification of various phenotypic traits such as visual organs, sting apparatus and ovaries (Peeters & Ito, 2015). Our results indicate that cuticle reduction is another parameter of this simplification. In general, Formicinae and Myrmicinae workers weigh much less than poneroid workers (Peeters & Ito, 2015). The inevitable end result of miniaturization is a decrease in phenotype quality. However, increasing the number of miniature 'autonomous units' brings benefits for life styles requiring a large foraging force (to hunt in group, scavenge dispersed carcasses or collect honeydew) or assembly-line tasks (e.g. fungus-farming). The evolutionary trend towards thinner cuticle documented here may be one of the reasons for the striking ecological success of ants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

- Appendix 1.** Collection details for the species that were sectioned. The number of specimens measured is given.
- Appendix 2.** Raw data for the species that were sectioned and measured. The ratio of cuticle thickness (in μm) divided by head width (in mm), multiplied by 1000, is also indicated.