Research

Scale insect (Hemiptera: Coccomorpha) morphology is transformed under trophobiosis

James Sodano[1,] [ID]**, Christopher E. Oufiero[1,]** [ID]**, Scott A. Schneider[2,]** [ID]**, John S. LaPolla[1,*,]** [ID]

[1]Department of Biological Sciences, Towson University, 8000 York Road, Towson, MD 21252, USA, [2]USDA, Agricultural Research Service, Henry A. Wallace Beltsville Agricultural Research Center, Systematic Entomology Laboratory, 10300 Baltimore Avenue, Beltsville, MD 20705, USA *Corresponding author, mail: jlapolla@towson.edu

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Ants (Hymenoptera: Formicidae) have great potential to exert influence over the morphological evolution of their obligate mutualist partners. Obligately myrmecophilic mealybugs are noted for their unusual morphology, and while this is often attributed to their relationship with ants, a quantitative assessment of this link is lacking. We address this need by evaluating morphological change among mealybugs as a function of ant association. This study considers the associates of 2 independent ant clades—*Acropyga* Roger, 1862 ants associated with root mealybugs from the families Xenococcidae and Rhizoecidae and herdsmen ants from the *Dolichoderus cuspidatus* (Smith, F., 1857) species-group associated with mealybugs from the tribe Allomyrmococcini (Pseudococcidae)—and compares them to free-living or potentially myrmecophilic species sampled from among the mealybugs and root mealybugs. We use a combination of geometric morphometric and linear datasets to evaluate characteristics of body shape, body size, leg metrics, and ostiole development. Obligate myrmecophily significantly influences both body shape and size. Myrmecophilous mealybugs are smaller than their free-living counterparts and are either pyriform or rotund in shape rather than oval. Ant-associates from Rhizoecidae also have significantly reduced anterior pairs of ostioles compared to free-living species. Ostioles are involved in defense against natural enemies and mutualist ants typically protect their partners, presumably supplanting the need for structures like ostioles among myrmecophilous species. We discuss the influence ants have on the evolution of their associates in the context of domestication and offer avenues for future exploration.

Key words: *Acropyga*, Coccoidea, morphometrics, mutualism, root mealybug

Introduction

Ants (Hymenoptera: Formicidae) engage in a myriad of mutualistic relationships with other organisms (Ness et al. 2006). Among some ants and their mutualistic partners, this has led to the evolution of ant agricultural systems, the best known of which is perhaps seen among the fungus-farming ants (Schultz 2021). But there is another side of ant agriculture that involves the herding of other insects, primarily scale insects (Hemiptera: Coccomorpha) and aphids (Hemiptera: Aphidoidea), in often elaborate relationships referred to as trophobiosis. In trophobiotic systems, herder ants provide the trophobionts protection from natural enemies in return for nutritive rewards through the collection of exudate called honeydew produced by the trophobionts. While many trophobiotic ants and trophobionts facultatively engage with each other, for a smaller group, the relationship is obligatory.

In obligatorily trophobiotic systems, ants have the potential to exert influence over the evolution of the species with which they partner. Trophobiotic ants can control the dispersal (Way 1963, Dill et al. 2002, LaPolla et al. 2002) and influence the reproduction (Ivens et al. 2012a, 2012b) of myrmecophilous partners, with evidence drawn from multiple systems. Interest in the dynamics of trophobiotic relationships has resulted in excellent reviews in recent years from Delabie (2001) and Ivens (2015). Having influence over dispersal and reproduction presents trophobiotic ants with an opportunity to impose extraordinary selective pressure on partnered species, which can result in morphological adaptation. Over time extreme selective pressure could potentially lead to the domestication

1

of partnered species here defined as the genetic modification of one species (the domesticate) by another (the farmer) in ways that benefit the farmer, but that would reduce the fitness of the domesticate in its original ecological niche (Schultz 2021). This leads to the question of whether there are any examples of trophobiotic ants that have domesticated their partners.

Root mealybugs from the family Xenococcidae possess several unique morphological traits thought to have arisen due to their obligate trophobiotic association with *Acropyga* Roger, 1862 ants. Their overall body shape is atypical for scale insects, being pyriform, often elongate, and with their abdomen distinctly curled over the dorsum, appearing scorpion-like (Fig. 1) (Balachowsky 1957, De Lotto 1977, Williams 1998, 2004b). In addition, xenococcids have strangely configured antennae with characteristic reduction of segmentation (Williams 1998, 2004b, Schneider and LaPolla 2011). They lack typical features that offer a degree of protection for free-living root mealybugs, such as wax pores and ostioles. While wax serves a variety of functions in scale insects, such as preventing water loss and preventing contamination from honeydew (Gullan and Kosztarab 1997), the complete loss of wax pores observed among xenococcids is unique. Perhaps because they no longer produce wax, they are covered in a dense layer of short setae over most of their body that may create a hydrophobic barrier (Williams 1978). Ostioles are thought to play an anti-predator role through reflexive bleeding and may release alarm pheromones (Williams

1978). Additionally, xenococcids have well-developed anal lobes with stout setae surrounding the anal pore; this forms a setal basket that suspends a droplet of honeydew in place until it can be taken up by an attendant ant. Way (1963) referred to this structure as the "trophobiotic organ" when morphological modifications for ant attendance were evident in scale insects and aphids. Silvestri (1924) , 1926) first described the unusual morphology of xenococcids and highlighted it as possibly relating to their mutual association with *Acropyga* ants. Early works by Bünzli (1935), Roba (1936), Weber (1944) and Flanders (1957) all emphasized the interdependence between mealybugs and *Acropyga* and morphological adaptations in both partners that likely evolved as a consequence of long-term interactions with one another.

Many of the unusual morphological traits found in Xenococcidae are analogous to traits found in more distant relatives from the tribe Allomyrmococcini (Pseudococcidae), the obligate myrmecophiles of a small group of *Dolichoderus* Lund, 1831 known as the migrating herdsmen ants. Like xenococcids, most allomyrmococcines have a pyriform-shaped body and are often densely coated in short setae. Both groups possess the trophobiotic organ. The antennae in both groups are also long and large relative to body size (although in the xenococcid genus *Eumyrmococcus* Silvestri, 1926 antennae are short) and their legs are well-developed. There are some notable discrepancies in their morphology too. Whereas xenococcids lack wax pores and ostioles, both are possessed by allomyrmococcines. Not


Fig. 1. *Eumyrmococcus sarnati* Schneider and LaPolla, 2011 females feeding on a root. (Photo: Eli Sarnat).

only are ostioles present in allomyrmococcines, but they are also distinctly enlarged and prominent (Williams 1978, Dill et al. 2002).

Acropyga and migrating herdsmen ants each have their respective groups of primary trophobiotic associates (Xenococcidae and Allomyrmococcini), however, at least among *Acropyga,* there are also secondary groups of myrmecophilous scale insects known to associate with them (Fig. 2). Multiple species of Rhizoecidae root mealybugs associate with *Acropyga* and it is clear that ant association has evolved several times independently among this group (Bünzli 1935, Delabie et al. 1991, Williams 1998, Tanaka 2016, Schneider and LaPolla 2020, 2022; unpublished phylogenetic results), in contrast to a single origin of association for the xenococcids (Schneider and LaPolla 2011). Furthermore, a species of Ortheziidae, a quite distant relative of mealybugs and root mealybugs (Gullan and Cook 2007, Vea and Grimaldi 2016), also associates with *Acropyga* (LaPolla et al. 2008). The morphology of this species is unusual compared to other ortheziids and shares some anecdotal similarities with *Acropyga*-associated rhizoecids and xenococcids (LaPolla et al. 2008). A number of other root mealybug species are speculated to associate with *Acropyga* as well but as yet lack confirmation of direct association with ants (see Schneider and LaPolla 2020, Schneider et al. 2022).

The *Acropyga*-scale insect and *Dolichoderus*-Allomyrmococcini mutualisms offer an opportunity to study morphological evolution under trophobiosis. The morphology of primary ant-associated lineages has putatively adapted in response to long-term obligatory association with ants, as described above. Might the same be true for secondarily associated groups? Have multiple independently evolving lineages that are associated with *Acropyga* (i.e., several rhizoecids and an ortheziid) undergone similar morphological transformations? And if so, is there evidence of a basic convergent body form common to myrmecophilous scale insects? Could such information be applied predictively to identify other species as myrmecophiles?

To our knowledge, no prior studies have evaluated this topic quantitatively. This study seeks to begin addressing such questions using a combination of geometric morphometrics and linear measurements to evaluate a set of morphological traits among associated, nonassociated (free-living), and potentially associated (speculative) species of root mealybugs (Rhizoecidae and Xenococcidae), mealybugs (Pseudococcidae), and an ensign scale (Ortheziidae). Specifically, this study addresses characteristics of overall body shape and size, leg size, and the development of ostioles.
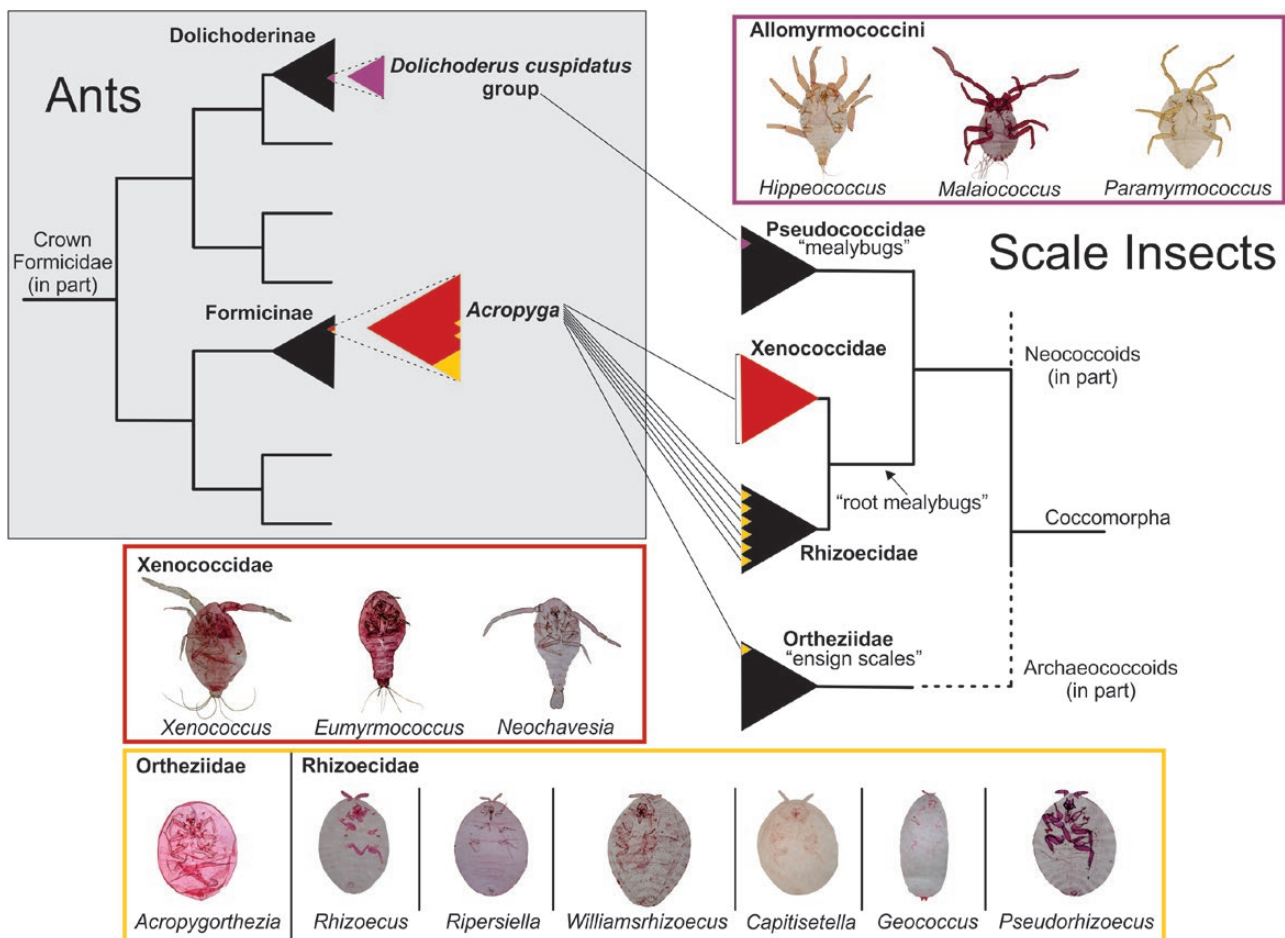
Fig. 2. A diagram of obligate associations considered in this study. Relationships among taxa are hypothesized based on evidence from published phylogenies of ants (modified from Blaimer et al. 2015, 2016) and scale insects (modified from Hardy et al. 2008, Vea and Grimaldi 2016, Choi and Lee 2022). Each triangle represents evolution of association. It is important to note that the number of times secondary associations have evolved among Rhizoecidae and *Acropyga* is almost certainly an underestimate as represented here (Schneider and LaPolla 2022). Images were captured with a 5× objective using a Zeiss AxioImage.M2 compound microscope and AxioCam imaging software.

Fig. 3. Queen *Acropyga goeldii* holding a root mealybug (likely *Rhizoecus* Kunckel d'Herculais, 1878 sp.) for her mating flight. (Photo: Alex Wild).

Materials and Methods

Study Systems

Acropyga and their trophobionts.

Acropyga ants are found pantropically, reaching their highest diversity in the Asian and New World tropics. There are presently 42 described *Acropyga* species (LaPolla 2004, LaPolla and Schneider 2023). *Acropyga* morphology suggests a completely subterranean existence because workers are relatively small (typically around 2 mm in total length) with reduced eyes (in some species only a single ommatidium remains), reduced antennal segmentation, a lightly pigmented cuticle, and setae covering their entire bodies. Workers also display photophobic behaviors when a nest is disturbed and they are never found foraging above ground. Nests are found in leaf litter, under stones, in rotten wood, and in the soil. *Acropyga* nests always contain trophobionts who presumably, through honeydew production, provide all the nutrition for the ant colony. All xenococcids (there are 34 known species from 3 genera: *Eumyrmococcus*, *Neochavesia* Williams & Granara de Willink, 1992 and *Xenococcus* Silvestri, 1924) are obligate trophobionts of *Acropyga* and they are co-distributed with *Acropyga* around the world (Schneider and LaPolla 2011, LaPolla and Schneider 2023).

By contrast, most rhizoecids are free-living (there are presently 223 rhizoecid species known) (García Morales et al. 2016); accessed 2 August 2023) with only a subset of species (at least 9 confirmed species) that are obligate trophobionts of *Acropyga* (Schneider and LaPolla 2022). While all rhizoecids produce wax, there is evidence that *Acropyga*-associated rhizoecids often produce less wax than free-living species (Schneider and LaPolla 2022). In fact, Schneider and LaPolla (2022) found that *Acropyga*-associated *Ripersiella* Tinsley, 1899 species lack tubular cerores, one of the structures used for wax production in rhizoecids. Nearly all other rhizoecids possess tubular cerores with few exceptions, some of which fall within the speculative list of associates (e.g., *Capitisetella migrans* (Green, 1933) and *Pseudorhizoecus proximus* Green, 1933) or associate with other ants (e.g., *Ripersiella malschae* (Williams, 2004) with *Pseudolasius* Emery, 1887). As for anti-predator structures like ostioles, among the *Acropyga*-associated rhizoecids Schneider and LaPolla (2022) suggested that the anterior ostioles were either lost or reduced in size. Outside of Rhizoecidae and Xenococcidae, there is one species of Ortheziidae (*Acropygorthezia williamsi* LaPolla and Miller, 2008) that has evolved an association with *Acropyga* as well (LaPolla et al. 2008). A second undescribed species of *Acropygorthezia* has been found but is at present only known from immature specimens (JSL and SAS, unpublished data).

Colony foundation is unique and occurs through trophophoresy. Unmated alate *Acropyga* queens vertically transmit their trophobionts by taking a single gravid individual (LaPolla and Spearman 2007) with them on their mating flights, holding the mealybug between their mandibles (LaPolla et al. 2002) (Fig. 3). The mealybug carried by the queen serves as a seed individual for a new colony of trophobionts in the new nest (Williams 1998, LaPolla 2004).

All specimens included in the study are adult females because this life stage serves as the foundation of scale insect taxonomy, and it is arguably the most critical life stage to consider in the context of trophobiosis. Adult females are relatively long-lived and all female instars are feeding stages, producing honeydew, which serves as the primary food source of associated ants (with the exception of an unusual female "pupal" stage that is unique to Xenococcidae) (Williams 1988, 1998). Conversely, males have fewer feeding stages, only the first 2 instars feed and produce honeydew, and they survive only briefly as adults (Williams 1998). Adult females are also the life stage that *Acropyga* queens transport during trophophoresy (LaPolla 2005, LaPolla and Spearman 2007, Smith et al. 2007, Tanaka 2016). Colony foundation is a critical juncture in the relationship between *Acropyga* and scale insects. The consequence of failure during colony foundation—such as a lost or crushed trophobiont—is death and total loss of fitness for both partners. Therefore, characteristics of fit between a scale species' body shape and the corresponding morphology of queens probably factor heavily in the success of colony foundation. We hypothesize that selective pressures for co-adaptation strongly influence the morphology of adult female myrmecophiles.

Dolichoderus and Allomyrmococcini.

Dolichoderus is a speciose genus of ants (over 130 described spp.) with a nearly global distribution, but notably absent from Africa. The twelve *Dolichoderus* species belonging to the *cuspidatus* (Smith, F., 1857) species-group are known as the migrating herdsmen

ants and are all obligately associated with Allomyrmococcini (Pseudococcidae) mealybugs. The *cuspidatus* species-group is restricted to southeast Asia, with Borneo the center of diversity for both the ants and their associated mealybugs. In contrast to *Acropyga*, herdsmen ants live above ground where they form bivouac nests and extensive trail networks; colonies frequently relocate (Dill et al. 2002). Herdsmen ant workers are large, typically over 4 mm in total length, and come in variable body colors from light yellow to black.

There are 25 species of allomyrmococcines from 10 genera, all of which are obligate trophobionts of migrating herdsmen ants. The trophobiosis between herdsmen ants and allomyrmococcines has been extensively studied and summarized by Dill et al. (2002).

Allomyrmococcines are transmitted to new ant colonies via colony fission (Dill et al. 2002). All herdsmen ants transport mealybugs in their mandibles, but some species also have mealybugs cling to their bodies during colony fission. It is also not unusual for mealybugs to stick together (due to their long body setae) when 1 mealybug is held in an ant's mandibles, thus making trophobiont transfer more efficient.

For the same reasons mentioned above, all specimens considered in this study are from the adult female stage.

Specimens Examined and Category Grouping

Images were generated from slide-mounted adult female scale insects for use in this study (Table 1). Specimens were obtained from the following collections: the U.S. National Museum of Natural History, housed at USDA ARS, Beltsville, MD, USA (USNM); The Natural History Museum, London, United Kingdom (BMNH); Museum National d'Histoire Naturelle, Paris, France (MNHN); Australian National Insect Collection, Canberra, Australia (ANIC); South African National Collection of Insects, Pretoria, South Africa (SANCI).

A total of 73 scale insect species were placed in 1 of 3 categorical groups regarding obligate association with ants: i) confirmed association, ii) speculative association, and iii) no obligate association known to occur (Table 1). These categories were used

to assess whether the morphological traits under study are significant predictors of obligate myrmecophily. Confirmed association for Ortheziidae, Rhizoecidae, and Xenococcidae indicates species are directly associated with *Acropyga* ants; confirmed association for Pseudococcidae indicates association with *Dolichoderus* ants. Confirmed trophobioses between species of *Acropyga* and scale insects are based on literature reports that conform to conservative standards for determining direct association discussed in Schneider et al. (2022). For Pseudococcidae, all members of Allomyrmococcini included in this study were considered obligatorily associated with *Dolichoderus* ants based on Dill et al. (2002). Speculative association refers to species of Rhizoecidae suspected of associating with *Acropyga* based on reports in the literature that have yet to be definitively confirmed; see Schneider and LaPolla (2020) for further discussion. Nonassociated species of Pseudococcidae and Rhizoecidae were included for comparison, but no speculative group of pseudococcids was considered. The nonassociated category can include species that associate with other ants facultatively, but they have no known relationships with *Acropyga* or herdsmen ants and their survival is not dependent upon association with ants.

Digitizing and Measurements

Prepared slide-mounted scale insect specimens were imaged in dorso-ventrally flattened positions on a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microscopy, LCC, White Plains, NY) with the aid of an AxioCam MRc digital camera and AxioVision v. 4.9.1 software. The specimen image was then reconstructed and rotated as needed to align the anterior region at the top of the image and the posterior region at the bottom using Photoshop (version 9.6.0.625). Some images were mirror-transformed in Photoshop so as to display the side of the specimen with less damage along the right side of the image. Image transformations allowed for consistent landmark placement and consistent specimen orientation. Specimen images were scaled proportionally to the actual size of the specimen using total length as an estimate for overall size. Images were then assigned a specimen ID and randomized to avoid human patterning errors during the placement of landmarks and semilandmarks.

Table 1. Species by association category: A list of species organized by their category of obligate ant association (associated, speculative, or no association) and family rank. The number of specimens sampled per species is included in parentheses following the name; the datasets in which each species were included are marked with a superscript as follows: the geometric morphometric dataset (G), the leg measurement dataset (L), and the ostiole measurement dataset (O)

Associated	**Ortheziidae:** *Acropygorthezia williamsi (3)*[G]
	Pseudococcidae: *Allomyrmococcus acariformis (1)*[G], *Archeomyrmococcus dolichoderi (1)*[L], *Bolbococcus oresbius (1)*[G], *B. sabahanus (2)*[GL], *Borneococcus bauensis (1)*[GL], *Dicranococcus sabahensis (1)*[GL], *Hippeococcus bundericus (3)*[GL], *H. wegneri (2)*[L], *Malaicoccus riouwensis (2)*[GL], *M. sarawakensis (1)*[GL], *Paramyrmococcus chiengraiensis (1)*[GL], *P. vietnamensis (2)*[L], *Thaimyrmococcus daviesi (1)* [GL]
	Rhizoecidae: *Rhizoecus (near) compotor (3)*[GLO], *Ripersiella campensis (3)*[GLO], *R. colombiensis (3)*[GLO], *R. illicians (3)*[GLO], *R. montanae (3)*[GLO], *R. pediandensis (3)*[GLO], *R. telalia (3)*[GLO], *Williamsrhizoecus udzungwensis (3)*[GLO]
	Xenococcidae: *Eurmyrmococcus adornocapillus (2)*[G], *E. corinthiacus (2)*[G], *E. lamondicus (1)*[G], *E. scorpioides (1)*[G], *E. sulawesicus (1)*[G], *E. williamsi (1)*[G], *Neochavesia caldasiae (4)*[GL], *N. cephalonodus (3)*[GL], *N. eversi (4)*[GL], *N. iwokramae (3)*[GL], *N. lapollai (6)*[GL], *N. linealuma (1)*[L], *N. podexuta (2)*[GL], *N. trinidadensis (4)*[GL], *N. weberi (3)*[GL], *Xenococcus acropygae (3)*[L], *X. baryglobosus (1)*[GL], *X. kinomurai (1)*[GL]
Speculative	**Rhizoecidae:** *Capitisetella migrans (3)*[GLO], *Geococcus coffeae (3)*[GLO], *Pseudorhizoecus proximus (3)*[GLO], *Rhizoecus arabicus (1)*[GL], *R. coffeae (3)*[GLO], *R. compotor (1)*[GLO], *R. mayanus (2)*[GLO], *Ripersiella andensis (3)*[GLO]
No Association	**Pseudococcidae:** *Dysmicoccus brevipes (3)*[GL], *Eurycoccus blanchardii (3)*[GL], *Ferrisia virgata (4)*[GL], *Planococcus citri (3)*[GL], *Pseudococcus longispinus (3)*[GL]
	Rhizoecidae: *Coccidella theobromae (3)*[GLO], *Rhizoecus apizacos (1)*[GLO], *R. associatus (3)*[GL], *R. atlanticus (3)*[GLO], *R. cyperalis (2)*[GLO], *R. divaricatus (3)*[GLO], *R. latus (2)*[GLO], *R. nemoralis (3)*[GLO], *R. nitidalis (3)*[GLO], *R. ovatus (2)*[GLO], *R. setosus (3)*[GLO], *R. simplex (3)*[GLO], *R. tropicalis (3)*[G], *R. variabilis (3)*[GLO], *Ripersiella campestris (3)*[GLO], *R. gracilis (3)*[GLO], *R. kondonis (3)*[GLO], *R. mexicana (3)*[GLO], *R. totonicapana (2)*[L], *Williamsrhizoecus epicopus (3)*[LO]

To capture scale insect shape, 2-D geometric morphometrics approach with thin-plate spline transformation (Rohlf 2010a: tpsDig version 2.32) was performed on a dataset of 167 images from 66 species (Table 1). Five digitized homologous landmarks were placed at fixed points along 1 side of the specimens' bodies. Landmarks were placed at fixed locations on each specimen at the following locations: i) at the anterior apex of the body evenly spaced between the antennae; ii) on the mesothorax in line with the articulation point of the second leg; iii) on the posterior lateral margin of abdominal segment 3; iv) on the posterior apex of the anal lobe; v) on the posterior margin of the anal pore (Fig. 4). Only one side of the insect was used for capturing the total body shape because mealybug specimens are often damaged during the slide mounting process, leaving unnatural bends, gaps, and other imperfections to the body curvature that may disrupt the shape and give misleading results. However, because scale insects are bilaterally symmetrical, this problem was circumvented by choosing the side of the body most intact for transforming an image into data points (i.e., for landmark analysis). In doing so, it was essential that the same side of each image be digitized in order for the subsequent analyses to run appropriately. For this reason, fixed homologous landmarks were always placed on the right side of the body. For specimens in which the right side of the body was too damaged, the image was mirrored along its central axis so that the left side could be used. Fixed landmarks were placed in the same order on each specimen starting with landmark 1 until reaching landmark 5.

After landmark placement, semilandmarks were placed between the fixed landmarks along the curvature of the specimen's body, connecting each landmark to the one that follows (Fig. 4). Semilandmarks were placed in the same order on each image and the same number of semilandmarks was used between each fixed landmark on each specimen image (between landmarks 1 and 2: 10 semilandmarks; between landmarks 2 and 3: 15 semilandmarks; between landmarks 3 and 4: 11 semilandmarks; between landmarks 4 and 5: 4 semilandmarks). Using tpsDig version 2.32, semilandmarks were evenly spaced from one another and then moved to the outline of the body, generating a smoother outline useful for accurately mapping the curvature of the body. Once all specimens were digitized in full, semilandmarks were converted to landmarks using tpsUtil (Rohlf 2010b: tps utility version 1.46), resulting in 45 total landmarks for each specimen image (5 homologous landmarks: 40 semilandmarks), and all data was read into R (R Core Team 2021: version 4.1.2) for subsequent analysis.

Linear measurements for body length and leg segment lengths and widths were obtained from a dataset compiled from 169 specimen images from 71 species (Table 1). Body length was measured from the most anterior point of the insect's body between the antennae to the most posterior point of the body between the anal lobes. All leg measurements (this included: trochanter, femur, tibia, tarsus, and tarsal claw) were performed on the metathoracic leg. Ostiole length measurements were compiled from 76 specimen images from 32 species of Rhizoecidae (Table 1). Ostioles were measured along their longest axis from the outer margins of the structure. All morphological features were measured in micrometers using AxioVision v.4.9.1 software.

Statistical Analyses

To determine root mealybug shape space, raw landmark data from digitized images were run through a generalized Procrustes analysis (GPA) using the R package *geomorph* (Collyer and Adams 2018, Adams et al. 2021, Baken et al. 2021: version 4.1.2). Because we had specific interests in comparing species within the Rhizoecidae, additional GPAs were executed on datasets removing the Xenococcidae and then also by removing the Xenococcidae and the Pseudococcidae.

A principal component analysis (PCA) on the GPA ordination of the datasets (i.e., all taxa, excluding xenococcids, and excluding xenococcids and pseudococcids) was used to assess body shape variation. Resulting PC scores were used to construct a morphospace occupied by each specimen performed on the GPA ordination of the datasets (i.e., all taxa, excluding xenococcids, and excluding xenococcids and pseudococcids). There were 86 total principal components generated, with the first 4 PCs explaining 82% of the variation. A MANOVA utilizing all 86 PCs was implemented to determine how successful the 3 established categories of association status (i.e., (i) confirmed association, (ii) speculative association, (iii) no association) are for predicting mealybug shape.
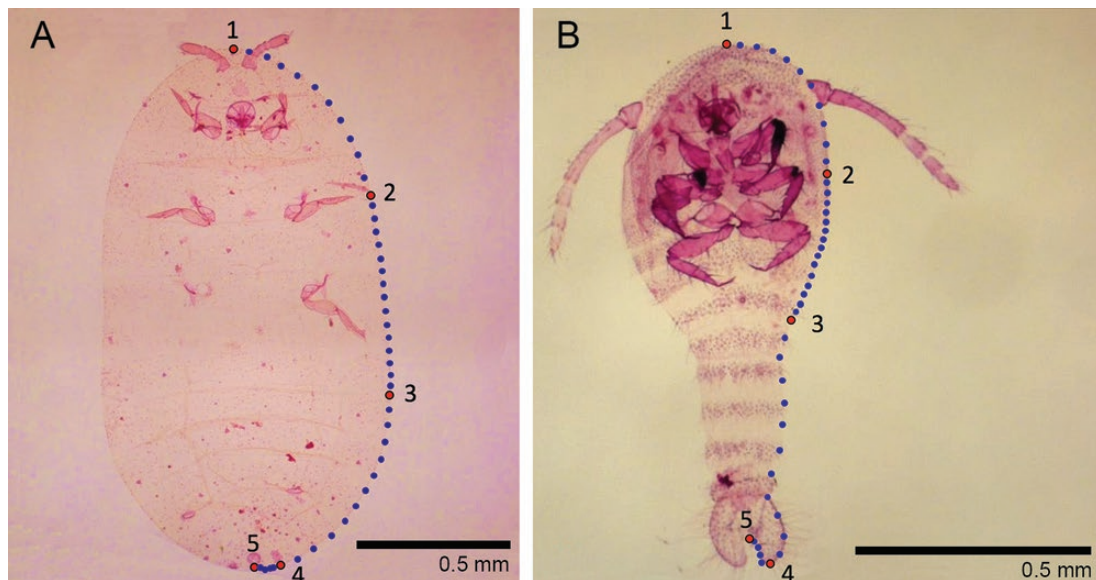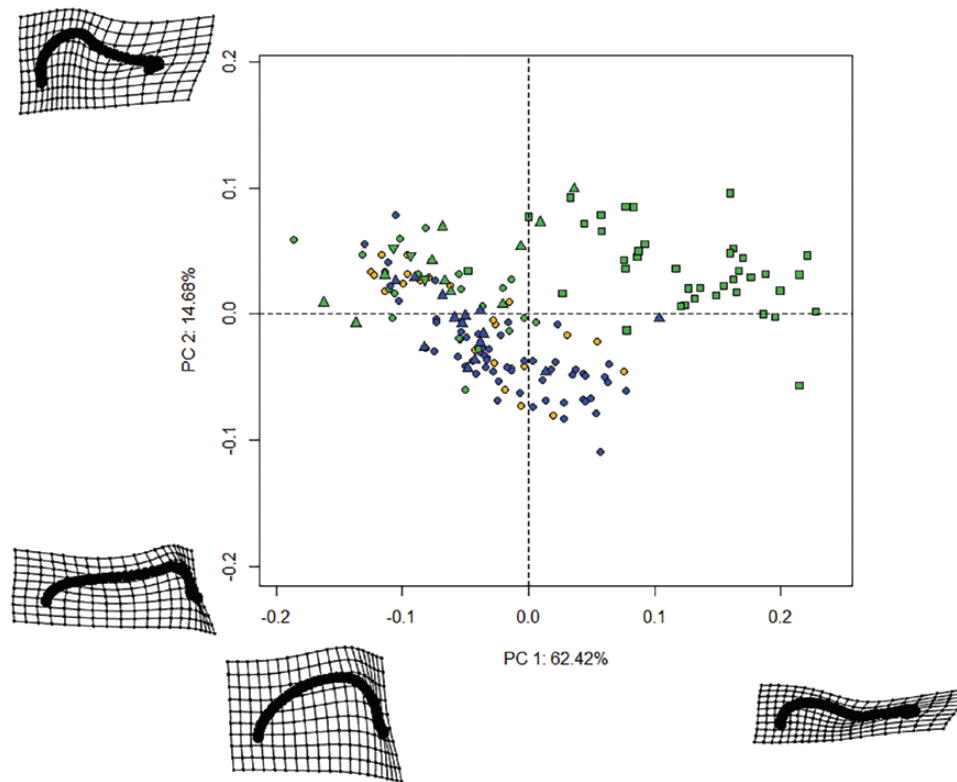

Fig. 4. Location of landmarks (numbered red dots) with semilandmarks placed between (blue dots). A) *Rhizoecus divaricatus* (USNM 75-10378) (example from Rhizoecidae), USNM; B) *Neochavesia trinidadensis* (holotype, USNM) (example from Xenococcidae).

Fig. 5. Generalized Procrustes analysis plots representing the scale insect consensus body shape. Anterior (a) and posterior (p) orientations are indicated along the *x*-axis.

For total body length, multiple comparisons were made between each mealybug lineage and their association status using an ANOVA. A Tukey's post hoc test was implemented following the ANOVA to provide between-group comparisons. A boxplot was generated from these analyses to visualize the results.

For leg trait data, linear regressions were completed to first examine how the length of each leg structure (i.e., trochanter, femur, tibia, tarsus, and tarsal claw) varied across the 3 categories of association in proportion to total body length. Additional regressions plotting structure width over body length were performed for the tibia and the femur. A PCA was performed on the standardized residuals from each regression of all specimens, coded by association status, to show which leg structure, if any, most differentiated ant-associated mealybugs from those that are free-living. We evaluated all 7 PCs generated and found that the first 4 PCs explained 93.2 % of the variation. We then used the broken-stick model to determine which PC axes to retain (Jackson 1993). This method compares the eigenvalues of each component against eigenvalues that are obtained from dividing the total variance randomly amongst the various components following a broken-stick distribution. If the observed eigenvalues exceed the eigenvalues generated from the broken-stick distribution, then they are considered interpretable. This method revealed that the first 4 PCs should be retained because their eigenvalues were higher than the corresponding random broken-stick components. Tukey's post hoc test was performed to once more compare between-group variation and determine if leg trait lengths differed significantly by association status.

Linear regressions were performed on ostiole data in the same way that leg trait data was treated above, including the use of residuals in analyses to correct for allometry. Using body length as a covariate, an ANCOVA was performed on both sets of ostioles to determine the effect of association status on the variation in ostiole length. Tukey's test provided between-group comparisons to determine if any category of association differed significantly from the others.

All linear data were handled in R using the packages dplyr (Wickham et al. 2022), mass (Venables and Ripley 2002), car (Fox and Weisberg 2019), factoextra (Kassambara and Mundt 2020), factoMinerR (Lê et al. 2008), and vegan (Oksanen et al. 2017).

Data Files

All data files and R scripts used in this study are available on Ag Data Commons: https://doi.org/10.15482/USDA.ADC/1529799.

Results

Morphometric Shape Variation

Three versions of the consensus body shapes were generated (Figs. 5–8) which revealed changes in consensus shape when (i) all the taxa are included, (ii) xenococcids were removed, and (iii) both xenococcids and pseudococcids were removed (Fig. 5). This allowed for consideration of the effect of taxon set on consensus shape. In the first analysis (including all taxonomic groups), the first 4 PCs for root mealybug shape space explained approximately 91% of the variation in the dataset, with PC1 and PC2 contributing to 87% of the variation (Fig. 6). PC1 accounts for variation along the length of the body, with positive PC values representing body shapes that are pyriform and elongated, and negative PC values representing circular body shapes (Fig. 6). PC2 accounts for shape variation in the width of the scale insects' bodies. Along this axis, positive PC2 values correspond to a wide anterior region that tapers off to a narrow posterior region, and negative PC2 values correspond to the opposite (narrower anterior and wider posterior) (Fig. 6). There is a clear separation dividing the xenococcids and some allomyrmococcines from the remaining groups (rhizoecids, ortheziids, and pseudococcids). This split is not surprising based on shared qualitative differences in xenoccocid and allomyrmococcine morphology (Williams 1978, 1998, 2004b, Kozár and Konczné Benedicty 2007, Schneider and LaPolla 2011), specifically having a pyriform-shaped body (see Xenococcidae and *Hippeococcus* Reyne, 1954 [Fig. 2]).

Our MANOVA analysis detected a highly significant relationship between association status and morphology ($F_{166} = 11.812$; $P = 0.001$). Associated scale insects were more likely to cluster around a similar morphospace, marked by rounded head regions and sharp tapering toward the posterior portion of the body. A wider, ovoid shape at both the anterior and posterior end was predicted for nonassociated scale insects, while the speculative association group ranged at both ends of the spectrum. However, these findings are likely to have been heavily influenced by the inclusion of Xenococcidae and Allomyrmococcini, which are all associated with ants and typically have a distinct pyriform body shape differing from that of the other taxa in our study. The ability to detect patterns in the other taxa based on our 3 categories of association is potentially obscured by the inclusion of xenococcids in particular, thus PCA was also run on a dataset excluding them (Fig. 7).

With the removal of xenococcids, the first 4 PCs captured 82% of the variation, with PC1 and PC2 explaining 76% of the variation (Fig. 7). PC1 describes variation in the body curvature, with positive values representing circular body shapes, and negative values

Fig. 6. Principal component analysis shape variation by association status with ants. Symbols are as follows: square = Xenococcidae; circle = Rhizoecidae; upward triangle = Pseudococcidae; downward triangle = Ortheziidae. Color indicates status with ants: green = confirmed obligatory association with ants; yellow = speculative obligatory association with ants; blue = no known obligatory association with ants. Confirmed association for Xenococcidae, Rhizoecidae, and Ortheziidae means unequivocal association with *Acropyga* ants; confirmed association for Pseudococcidae means association with *Dolichoderus* ants; speculative association refers to Rhizoecidae reported to associate with *Acropyga* but their relationships have not been confirmed. Wireframes depict the variation in morphospace captured along each PC axis.

representing elongate-oval body shapes (Fig. 7). Along the PC2 axis, positive values correlate to narrower anterior regions and wider posterior regions, reflecting a pear shape, while negative PC2 values depict the opposite, with wider anterior regions and narrower posterior regions. The PCA plot shows a largely distinct separation in the morphospace occupied between associated and nonassociated scales (Fig. 7). Confirmed associated rhizoecids and allomyrmococcines cluster in the morphospace along the positive PC1 axis, while being quite varied along PC2, demonstrative of rounder bodies that vary in width at the posterior and anterior ends. Nonassociated rhizoecids and pseudococcids are shown clustering on the negative PC1 axis and are generally tighter to the zero line on PC2, indicative of elongate-oval bodies that do not taper toward either the anterior or posterior end. Once again, the speculative group varied between all regions of the morphospace. A MANOVA performed on randomized raw values for this ordination was used, which detected a highly significant relationship between association status and morphospace (F_{127} = 11.398; P = 0.001).

A final PCA was run while excluding both xenococcids and pseudococcids to allow for a more focused study of shape in rhizoecids only. For this dataset, the first 4 PCs captured 86% of the variation, with PC1 and PC2 accounting for 82% of the total variation (Fig. 8). The PC axes for this PCA are nearly identical to those seen in Fig. 7, with PC1 representing elongate to oval body shapes and PC2 describing variation in the body width at the posterior and anterior ends respectively. A clear separation between associated rhizoecids and free-living rhizoecids is observed. The 3 ortheziid

specimens (*Acropygorthezia williamsi*, associated with *Acropyga myops*), group tightly within the cluster of associated rhizoecids on the PCA plot, suggestive of shape convergence in associated scale insects from 2 distantly related families (Cook et al. 2002, Hardy et al. 2008, Vea and Grimaldi 2016). Once again, a MANOVA was performed for this ordination, which detected a highly significant relationship between association status and morphospace (F_{102} = 12.383; P = 0.001).

Body Length Analysis

Figure 9 depicts scale insect body length in relation to the 6 different association status categories among taxonomic groupings (pseudococcid associated, pseudococcid nonassociated, rhizoecid associated, rhizoecid speculative, rhizoecid nonassociated, and xenococcid associated). A Tukey's post hoc test, indicated 4 statistically significant groups found based on body size. Tellingly, these groups correlate with association status of the scale insects. Within the pseudococcids, 2 distinct groups were found based on differences in body size, with associated pseudococcids having statistically smaller body sizes than the nonassociated pseudococcids (P = 0.01). A similar result was found in root mealybugs, with the associated and speculatively associated rhizoecids grouping with the xenococcids based on having similarly small body sizes compared to nonassociated rhizoecids (>0.05 for stepwise comparisons between associated rhizoecids, speculative rhizoecids, and xenococcids; P < 0.05 for stepwise comparisons between nonassociated rhizoecids and the previously stated 3 groups). Overall, the analysis found that
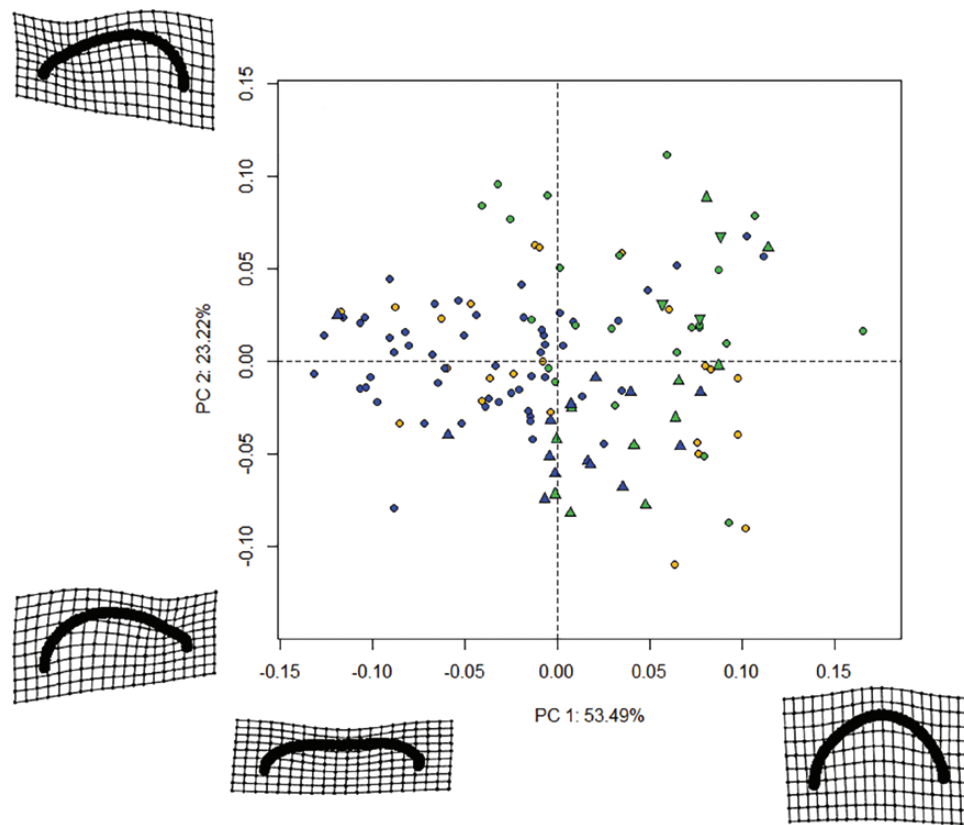
Fig. 7. Principal component analysis shape variation by association status with ants excluding Xenococcidae. Symbols are as follows: circle = Rhizoecidae; upward triangle = Pseudococcidae; downward triangle = Ortheziidae. Color indicates status with ants: green = confirmed obligatory association with ants; yellow = speculative obligatory association with ants; blue = no known obligatory association with ants. Confirmed association for Rhizoecidae and Ortheziidae means association with *Acropyga* ants; confirmed association for Pseudococcidae means association with *Dolichoderus* ants; speculative association refers to Rhizoecidae reported to associate with *Acropyga* but their relationships have not been confirmed. Wireframes depict the variation in morphospace captured along each PC axis.

associated scale insects tend to have a smaller overall body size compared to their free-living, nonassociated relatives.

Linear Leg Analysis

For each of the 5 structures of the leg that we measured, linear models were generated showing a positive proportionality between leg trait size and body length. Across all taxa and association classes included in this study, longer leg structures generally belonged to larger scale insects. For the femur and tibia, width was also measured on each specimen, having the same positive relationship with body size as length.

From each of these regression models, the standardized residuals for each measurement were obtained to allometrically scale leg structures to body size. Seven PCs were generated with the first 2 PCs explaining 75.7% of the total variation. Of the 5 categorical groups, xenococcids were the only group to have a prominent spread along PC2. However, after applying a broken-stick model to the first 4 PCs, only the first PC (length) showed a variation greater than what was to be expected based on random chance. An ANOVA on PC1 in leg trait length among our 5 groups is significant ($F_4 = 6.169$; $P = 0.0001$). A subsequent Tukey's post hoc test revealed that only the allomyrmococcines had significantly longer legs than all other groups (P-values ≤ 0.001 for all stepwise comparisons involving allomyrmococcines).

Ostiole Size

For both anterior and posterior ostioles, linear regressions plotting ostiole diameter length over insect body length revealed significant

positive relationships respectively ($P < 0.001$) (Fig. 10). On each regression, zero values were added to account for those specimens in which ostiole pairs are absent. For anterior ostioles, the ANCOVA indicated that association status did have a significant impact on the length of the ostiole after body size had been accounted for as a covariate ($F_2 = 5.0484$; $P = 0.009$). Running Tukey's post hoc test denoted that the strongest differences occurred between the confirmed associated and the free-living groups ($P < 0.001$). Additionally, a significant difference was also found between free-living and speculatively associated rhizoecids ($P = 0.0001$), while the confirmed association and speculative group showed no statistical difference from one another ($P > 0.05$). The ANCOVA on posterior ostiole length and association status found that there was no statistically significant relationship between these 2 variables ($P = 0.281$). Likewise, Tukey's post hoc found no statistical differences between any of the 3 established groups of association status ($P > 0.05$). This suggests that association status in rhizoecids does not influence posterior ostiole length.

Discussion

Engaging in obligatory trophobiotic association with ants can transform the morphology of scale insects. While there has long been an interest in myrmecophilous scale insects and morphological traits that may arise as adaptations to trophobiosis (Bünzli 1935, Way 1963, Williams 1978, 1998, 2004b, Gullan and Kosztarab 1997),
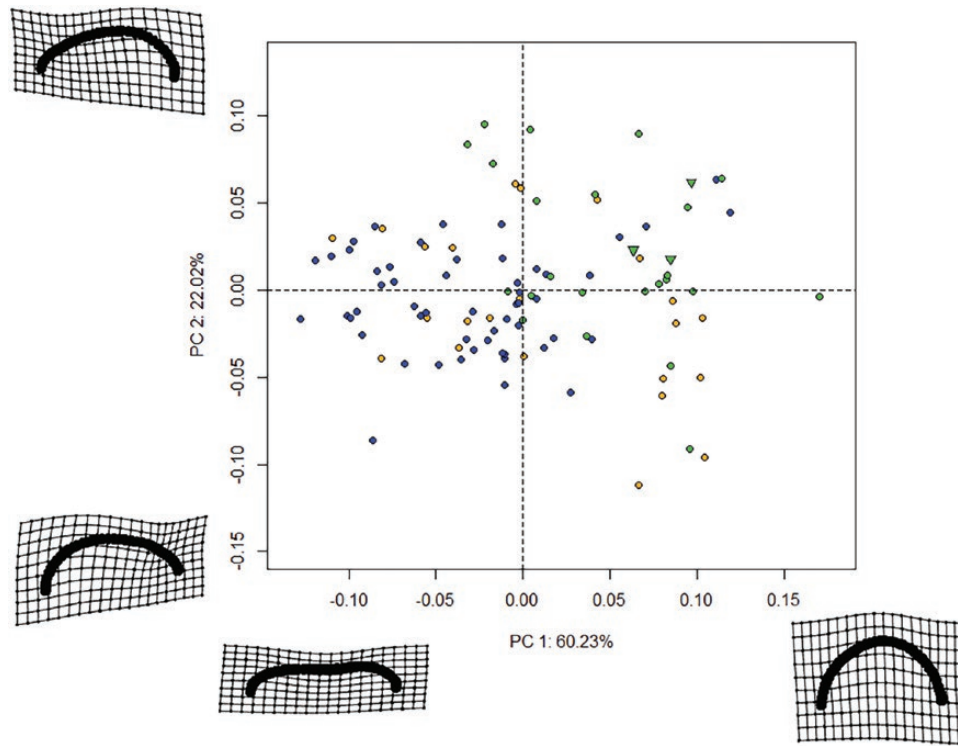
Fig. 8. Principal component analysis shape variation by association status with ants excluding Xenococcidae and Pseudococcidae. Symbols are as follows: circle = Rhizoecidae; downward triangle = Ortheziidae. Color indicates status with ants: green = confirmed obligatory association with *Acropyga*; yellow = speculative obligatory association with *Acropyga*; blue = no known obligatory association with *Acropyga*. Wireframes depict the variation in morphospace captured along each PC axis.
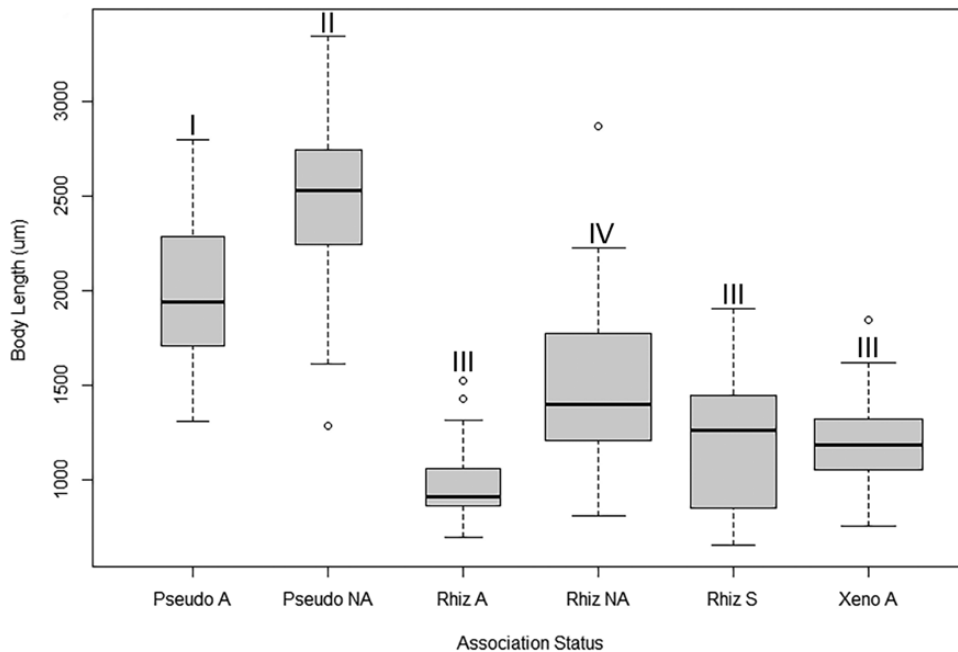
Fig. 9. Boxplot of mealybug body length categorized by taxonomy and association status with ants. Pseudo A = Pseudococcidae associated with *Dolichoderus*; Pseudo NA = not associated with *Dolichoderus*; Rhiz A = Rhizoecidae associated with *Acropyga*; Rhiz NA = Rhizoecidae not associated with *Acropyga*; Rhiz S = Rhizoecidae speculatively associated with *Acropyga*; Xeno A = Xenococcidae; all species are associated with *Acropyga*. Roman numerals above each group represent groups that are statistically the same. One circle indicates *P*-value = 0.05; 2 circles indicate *P*-value = 0.01.

this study is the first to quantify such traits. Our results show that myrmecophilous scale insects from multiple lineages have converged on similar morphologies even when associating with different groups of ants. They significantly differ in both body size and shape compared to free-living relatives—being consistently smaller and either pyriform or circular in shape (Figs. 2 and 11). Furthermore,
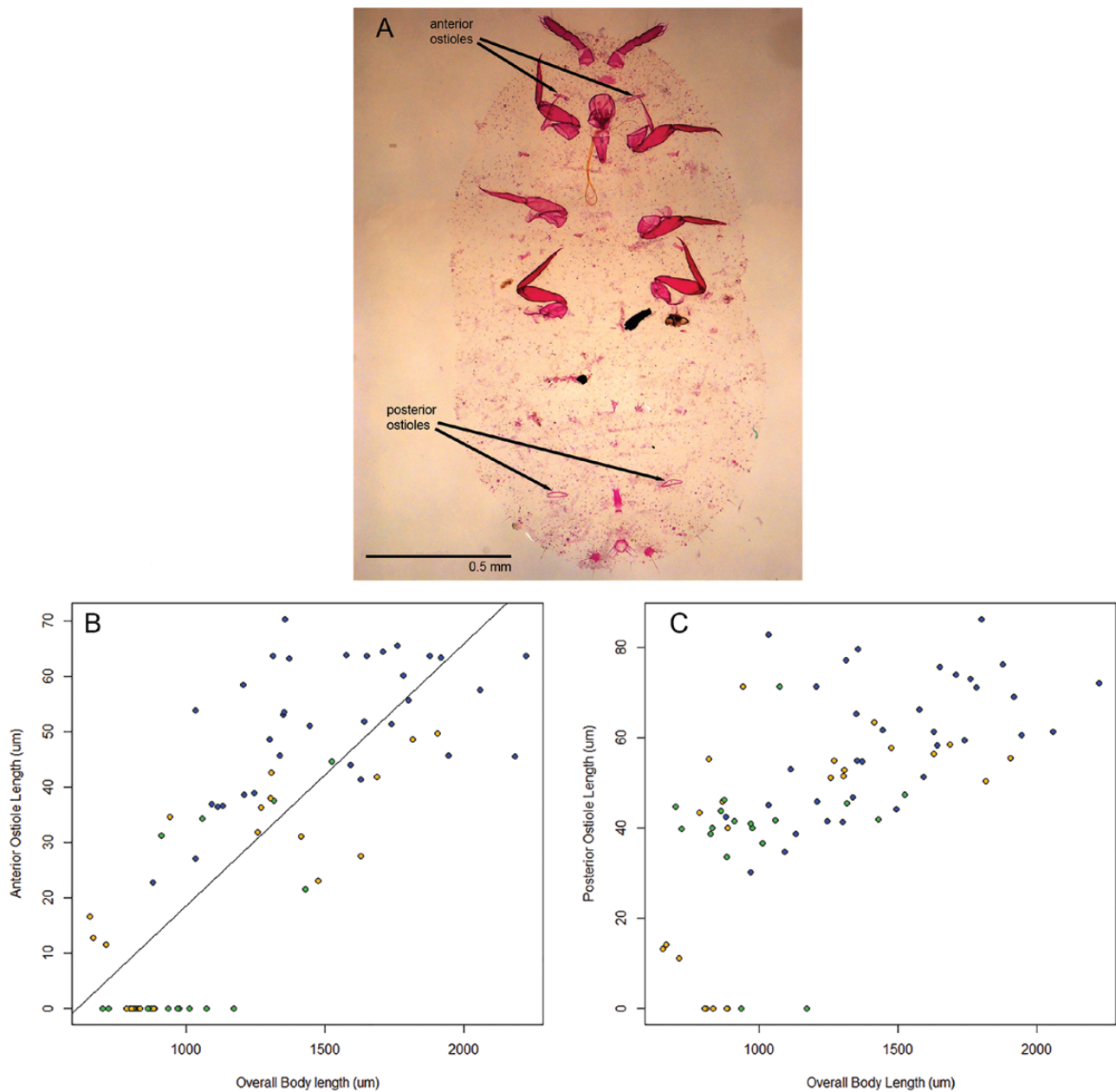
Fig. 10. A) Showing positions of ostioles on a rhizoecid root mealybug (shown is the holotype specimen of *Rhizoecus nitidalis*). Linear regression plots for ostiole size over body length of rhizoecids: B) anterior ostioles; C) posterior ostioles. Color indicates status with ants: green = confirmed obligatory association with *Acropyga*; yellow = speculative obligatory association with *Acropyga*; blue = no known obligatory association with *Acropyga*.

associated root mealybugs have either lost their ostioles entirely or ostioles have become significantly reduced in size, trending toward loss. Evidence from this study demonstrates that the morphology of mealybug lineages (with examples from Pseudococcidae, Rhizoecidae, and Xenococcidae) changes in consistent ways in response to long-term obligatory association with ants. These patterns extend even further to include more distantly related scale insects, such as ensign scales (Ortheziidae).

Morphometric analysis revealed that 2 classes of overall body shape have evolved among *Acropyga*-associated scale insects: pyriform bodies are characteristic of the primary associates (Xenococcidae) and circular bodies, often constricted near the head or prothorax, are found among the secondary associates (Rhizoecidae and Ortheziidae) (Fig. 6). Fascinatingly, these same 2

classes of body shape also characterize the Allomyrmococcini, associated with *Dolichoderus* ants. Therefore, multiple independently evolving groups of obligate trophobionts, from 4 scale insect families, associating with 2 groups of ants (from 2 different subfamilies: Formicinae and Dolichoderinae) have all converged on the same 2 body shape classes.

Our data show that trophobionts are small-bodied compared to their free-living relatives (Figs. 9 and 11), and the recurrence of this result among independent mutualist groups suggests that this is selective. Myrmecophiles associated with *Acropyga* and *Dolichoderus* are smaller than their free-living relatives from Rhizoecidae and Pseudococcidae. In fact, all the *Acropyga* associates form a statistically significant group, smaller than free-living rhizoecids to which they were compared (Fig. 9). And while pseudococcids are generally
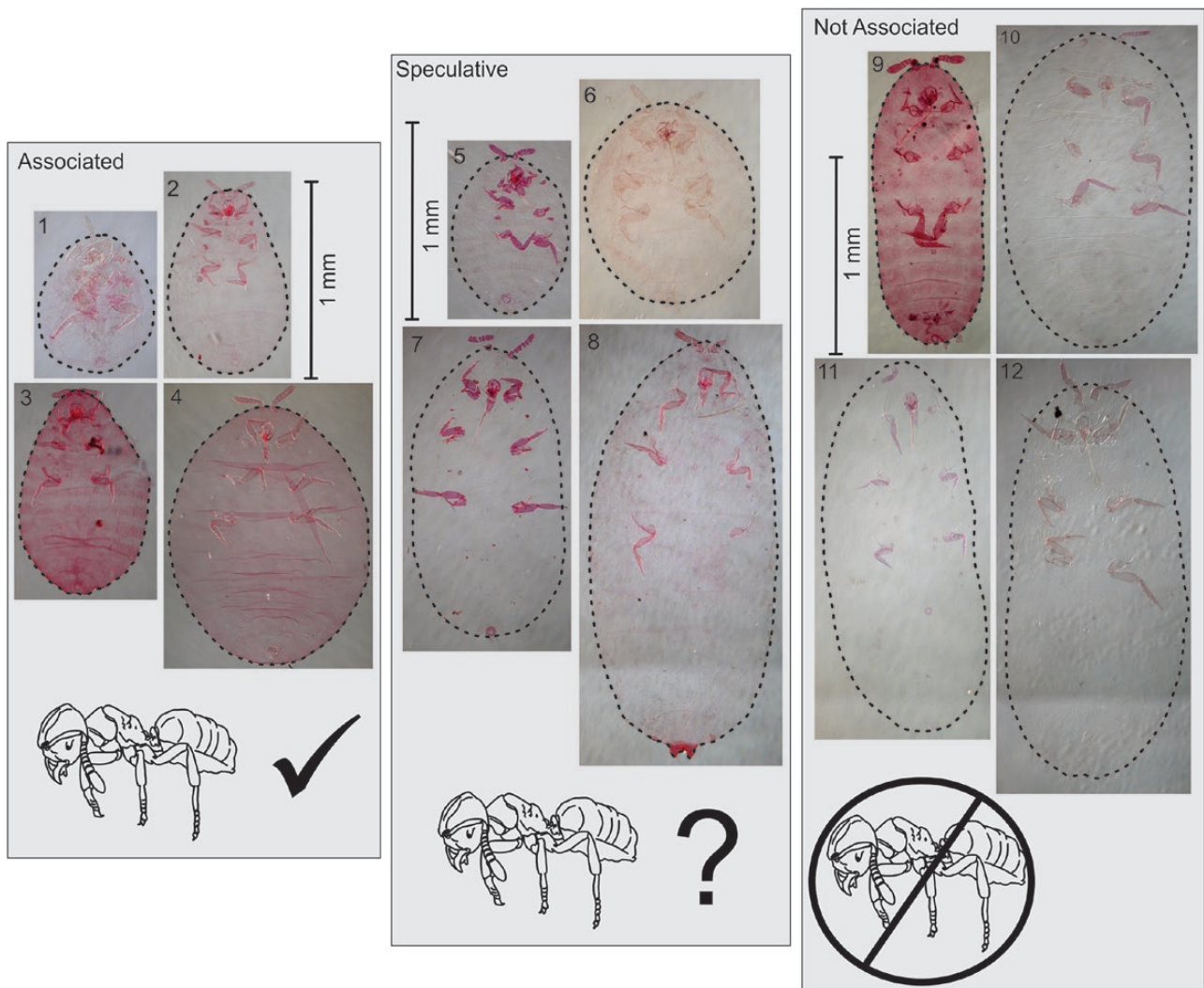
Fig. 11. Slide-mounted root mealybugs by association category. Examples of 4 adult female specimens each from 3 categories of association with *Acropyga*— Associated, Speculative, and Not Associated—with their body outlined. Images were captured with a 5× objective using a Zeiss AxioImage.M2 compound microscope and AxioCam imaging software. The following species are presented to scale: 1. *Ripersiella colombiensis* (Hambleton, 1946), 2. *Ripersiella campensis* Schneider and LaPolla, 2022, 3. *Ripersiella montanae* Schneider and LaPolla, 2022, 4. *Ripersiella pediandensis* Schneider & LaPolla, 2022, 5. *Rhizoecus compotor* Williams & Granara deWillink, 1992, 6. *Capitisetella migrans* (Green, 1933), 7. *Rhizoecus arabicus* Hambleton, 1976, 8. *Geococcus coffeae* Green, 1933, 9. *Ripersiella campestris* (Hambleton, 1946), 10. *Rhizoecus cyperalis* (Hambleton, 1946), 11. *Ripersiella kondonis* (Kuwana, 1923), 12. *Ripersiella mexicana* Hambleton, 1946.

larger than root mealybugs overall, the allomyrmococcines are statistically smaller than free-living pseudococcids (Fig. 9). It would be interesting to determine how universal this principle is among other myrmecophilous scale insects. Trophobionts must be carried by worker ants, which likely constrains their body size. Size selection is probably acute among *Acropyga* given that unmated queens must fly from their birth nest carrying a trophobiont in their mandibles. Although the biomechanics of trophophoresy has not yet been studied, presumably there are limits to the size of a root mealybug and a queen's ability to carry them in flight.

One of the defining morphological features of the Xenococcidae is the absence of ostioles. These structures provide protection against natural enemies (such as predators and parasitoids) through reflexive bleeding (Williams 1978), and the absence of ostioles among xenococcids arguably results from their relationship with *Acropyga* (Williams 1998, Schneider and LaPolla 2011). Among *Acropyga*-associated rhizoecids, the anterior ostioles are either completely absent or, when present, are significantly shorter than those in free-living

species (Fig. 10). Thus, for root mealybugs in general, an obligatory relationship with ants results in the loss or reduction of ostioles. Myrmecophilous root mealybugs rely on ants for defense against natural enemies (Schneider and LaPolla 2022) in lieu of relying on defensive structures like ostioles. As noted by Schneider and LaPolla (2022), *Acropyga*-associated root mealybugs lack other structures that would normally protect them either from natural enemies or from abiotic factors such as desiccation, further signaling their dependence on ants for survival. For example, all associated rhizoecids—as well as several speculatively associated species—lack wax-producing structures called tubular cerores, but nearly all free-living rhizoecids possess them (Kozár and Konczné Benedicty 2007). Furthermore, though all associated rhizoecids still possess some types of wax pores (in contrast to xenococcids), there is observational evidence that the amount of wax they produce is less than that found in free-living species (Schneider and LaPolla 2022). Therefore, the reduction/loss of anterior ostioles, loss of tubular cerores, and decreased wax production are all features corresponding to ant association.

The combined evidence of convergence in body shape, body size, and modification of ostioles discovered here indicates that mutualist ants influence the morphological evolution of obligate trophobionts. By forming associations with multiple lineages of scale insects, *Acropyga* have engaged in a type of natural experiment that illustrates the elements of domestication for mealybugs. It is apparent that trophobiont morphology has transformed in consistent ways among multiple lineages, genera, and even families of scale insects in response to ant association. And, as further proof of concept, the relationship between Allomyrmococcini and migrating herdsmen ants has demonstrated that certain morphological traits will converge among myrmecophilous mealybugs even when the ants they associate with are distant relatives. To drive this point home, consider 1 final group of myrmecophilous rhizoecids, this time associated with *Pseudolasius* ants on *Macaranga* Thouars, 1806 (Euphorbiaceae) in Malaysia ([Williams 2004a](#)). Four species that were not considered in the present analyses, *Geococcus anthocomus Williams, 2004*, *Rhizoecus pseudolasii* Williams, 2004, *Ripersiella gombakensis* (Williams, 2004), and *R. malschae*, each show the elements of domestication through myrmecophily illustrated above. Three of the 4 species have pyriform-shaped bodies, the fourth (*G. anthocomus*) is broadly circular in shape. In 3 of these species, the anterior ostioles are absent; in the fourth species (*R. gombakensis*) they are present but ill-defined. And although tubular cerores are present in 3 of these species, one of them (*R. malschae*) has lost cerores entirely. Two of the species (*R. pseudolasii* and *R. malschae*) also have dense patches of setae, much like the xenococcids and allomyrmococcines. These species belong to 3 separate genera, associate with yet another group of distantly related ants, and all converge on the same set of atypical morphological traits seen among the associates of *Acropyga* and *Dolichoderus*. The common theme linking all the scale insects discussed here is obligate myrmecophily.

Can the principles illustrated here be applied predictively to identify or confirm myrmecophily among other scale species? The answer, for now, must remain—maybe. Further investigation into this question seems promising and could be useful when considering the speculative group of associates included in our analyses. Given the results regarding associated species, one might feel confident that *Capitisetella migrans* and *Rhizoecus compotor* are directly associated with ants, and perhaps less confident regarding the association of *Geococcus coffeae* and *Rhizoecus arabicus* ([Fig. 11](#)). There is potential to apply this in a rigorous fashion to address other species for which information about their natural history is lacking. For example, *Ripersiella sepilokensis* is a species of Rhizoecidae known from leaf litter samples collected in Malaysia. It has a broadly circular body, is entirely lacking ostioles, and is densely coated in setae. In his description, [Williams (2004b)](#) even commented that this species probably lives in close association with a species of ant. Perhaps the findings of the present study bring us a step closer to knowing for certain.

Does this mean the examples of obligate myrmecophily investigated here represent cases of domestication by ants? The definition for domestication following [Schultz (2021)](#), emphasizes that the ants (the farmers) would need to have caused genetic modification in the scale insects (the domesticates) that would benefit the ants, but reduce the fitness of the scale insects if they were free-living to show evidence of domestication. Among the general morphological characteristics found in scale insects to correlate with *Acropyga*-association (pyriform or circular body shape, reduced body size, and reduction/loss of ostioles), the reduction/loss of wax-producing structures such as tubular cerores and wax pores and the reduction/loss of defensive structures such as ostioles present the best case for loss of fitness

if those mealybugs were still free-living. Is this enough to say that *Acropyga* have domesticated their trophobionts? Perhaps, but before we make claims of domestication we would like to see further investigation into other morphological traits to see if they correlate with myrmecophily. Is there a morphological syndrome that can be identified for *Acropyga*-associated scale insects (in addition to features such as body shape and ostiole reduction discussed in this study)? Additionally, explicit studies of the genetic structure of *Acropyga*-associated scale populations could reveal the role important events such as trophophoresy play in influencing the genetic makeup of the scale insects themselves. For example, trophophoresy could potentially act as a powerful genetic bottleneck with each reproductive episode. The issue of domestication aside, what we can say for certain is that obligate trophobiosis does influence the morphology of the species ants partner with in profound ways, showing these relationships are both deep and enduring.

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Author Contributions

James Sodano (Data curation [Lead], Formal analysis [Lead], Investigation [Equal], Methodology [Equal], Software [Equal], Validation [Lead], Visualization [Lead], Writing—original draft [Equal], Writing—review & editing [Equal]), Christopher Oufiero (Conceptualization [Supporting], Formal analysis [Supporting], Methodology [Supporting], Software [Supporting], Writing—original draft [Supporting], Writing—review & editing [Supporting]), Scott Schneider (Conceptualization [Equal], Data curation [Supporting], Formal analysis [Supporting], Funding acquisition [Equal], Investigation [Equal], Methodology [Supporting], Resources [Equal], Supervision [Equal], Validation [Supporting], Visualization [Equal], Writing—original draft [Equal], Writing—review & editing [Equal]), and John LaPolla (Conceptualization [Lead], Data curation [Equal], Formal analysis [Supporting], Funding acquisition [Equal], Investigation [Equal], Methodology [Equal], Project administration [Lead], Resources [Equal], Software [Supporting], Supervision [Lead], Validation [Supporting], Visualization [Supporting], Writing—original draft [Equal], Writing—review & editing [Equal])

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