Myrmecological News
ISSN 1997-3500
myrmecologicalnews.org
Myrmecol. News 31: 201-216 doi: 10.25849/myrmecol.news_031:201 6 October 2021
Original Article

# Hairs distinguish castes and sexes: identifying the early ontogenetic building blocks of a fungus-farming superorganism (Hymenoptera: Formicidae)

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**Abstract**

Ants are among the best-known insects, but the morphology and development of their larvae are rarely studied in a systematic manner. Precise information on larval development is needed not only to understand ontogenetic development of caste phenotypes but also ultimately to allow a better understanding of the integrated development of entire ant colonies – superorganisms that have an inseminated founding queen as germ-line, cohorts of unmated workers as soma, and the iteroparously produced gyne and male reproductives as gamete analogues. Here, we present a survey of larval morphology of the fungus-growing ant *Acromyrmex echinatior* (Forel, 1899), documenting the four instars of large and small workers and the five instars of gyne and male larvae. We used a combination of quantitative traits (body length, body curvature, hair patterning, head to body length ratio) and binary traits (presence / absence of anchor-tipped hairs, gut full / empty, head moving or not), and we document variation across the instars and sexes for 251 individuals with z-stacked images. Based on the statistical resolution of single and combined traits, we provide a key for the 3rd to 5th instar larvae, where sex and developmental stage can be unambiguously identified, and offer notes on the second instar, where identifications are statistically possible but with lower accuracy. This key is also available as an electronic resource <https://megalomyrmex.osu.edu/apps/acro-larva-key/>. We discuss the challenges involved in this type of research and highlight opportunities for addressing new research questions that become accessible when sex-specific and caste-specific larval instars can be distinguished.

**Key words:** *Acromyrmex echinatior*, leaf-cutting ants, larval development, z-stack, taxonomy.

Received 1 May 2021; revision received 2 August 2021; accepted 11 August 2021
Subject Editor: Manuela de Oliveira Ramalho

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## Introduction

All ant species have colonies with physically differentiated castes. This primarily reflects profound division of labor between specialized inseminated queens and unmated workers but sometimes also involves morphologically distinct soldiers or other specialized castes (Wheeler 1910, 1928, Hölldobler & Wilson 1990). Exceptions to this rule are few and apparent because they are evolutionarily derived reductions of more typical ancestral caste phenotypes (Peeters & Ito 2015, Boomsma & Gawne 2018, Peeters 2019). These fundamental characteristics have given the ants a center stage in evolutionary biology ever since Darwin (1859). He observed that the ants must

have originated and differentiated by selection at the "family" level, alluding to "the neuters or sterile females in insect communities: for these neuters often differ widely in instinct and in structure from both the males and the fertile females, and yet, from being sterile, they cannot propagate their kind." A few decades later, Weismann (1893) used worker sterility of ants as a strong argument against Lamarckian inheritance because the adaptations of sterile castes could not be due to acquired characters when they cannot reproduce. The same logic led Wheeler (1911) to propose that ant colonies represent a higher level of metazoan organismality because single-queen colonies have a germ line and soma and a clear ontogeny; a concept for which he later coined the term superorganism to emphasize that ant colonies represent a level of organizational complexity that is analogous to, but irreversibly beyond, that of an animal body (Boomsma & Gawne 2018).

The ontogeny of animal bodies is increasingly well understood. The field that used to be referred to as comparative embryology has been revitalized into modern developmental biology, or evo-devo, which explicitly considers evolutionary aspects (Carroll 2008). However, the developmental biology of ant superorganisms is still in its infancy. This is understandable in part because ant larvae are not fixed in cells as in honey bees or yellow jacket wasps, so making timed "cross sections" through ant colonies in different stages of collective ontogeny is a daunting task. However, the number of larval worker instars has been determined for at least 64 ant species (reviewed in Solis & al. (2010)). It was decades ago that Oster & Wilson (1978) suggested that a typical ant colony goes through a founding stage, an ergonomic stage, and an iteroparous reproductive stage, but we remain largely clueless about the gene regulation networks that characterize these stages. At the same time, following the development of the individual ant larvae that will ultimately make up the adults of a colony is challenging because individuals are frequently moved around by nurse workers. Noteworthy pioneering studies did happen though. Brian and co-workers followed larval development of *Myrmica* ants in the 1950s and 1960s, mapping the molts and instars of female castes and showing that workers can actively inhibit large larvae from developing into gynes (Brian 1951, Brian & Kelly 1967, Brian 1975). A few decades later, Diana Wheeler contributed pioneering studies on worker polymorphism (Wheeler 1991) and noted that there may be multiple aspects of evolutionary conflict over larval development; an idea further elaborated by Bourke & Ratnieks (1999).

In recent years, several genetic and physiological mechanisms that affect larval development and caste differentiation in ants have become elucidated (Abouheif & Wray 2002, Rajakumar & al. 2012, Libbrecht & al. 2013, Schrader & al. 2015, 2017), and rudimentary wing discs were shown to be key in facilitating the evolution of complex caste systems (Rajakumar & al. 2018, Oettler & al. 2019). However, we are still lacking a deeper understanding of the principles that govern phenotypic plasticity and developmental canalization of alternative caste phenotypes (but see Klein & al. 2016, Nagel & al. 2020). Against this background, we set out to explore the potential of the fungus-growing ant *Acromyrmex echinatior* (Forel, 1899) as a model for studying the developmental biology of ant superorganisms, which requires proper characterization of all categories of immature ants. There are several reasons for focusing on this ant: (1) Its genome has been sequenced and annotated (Nygaard & al. 2011, Qiu & al. 2018) and will soon exist in chromosome-level resolution (Boomsma & al. 2017), and many genomes of fungus-growing ant species from other genera (Formicidae: Myrmicinae: Attini: subtribe Attina; hereafter "attine" ants) are also available (Nygaard & al. 2016); (2) its queens can live for up to two decades and brood can be sampled repeatedly from the same captive colonies (Nehring & al. 2018); (3) male and female reproductives are readily produced in captive colonies (Dijkstra & Boomsma 2008); (4) founding queens can be collected in the field and be established in lab culture (Howe & al. 2019); (5) colonies have three distinct castes (small workers, large workers, gynes / queens) allowing comparisons across larval developmental pathways that ultimately give rise to three very different adult phenotypes (Hughes & al. 2010); and (6) colony size and body size of individuals are large enough to allow both destructive sampling of substantial numbers of ants and feasible dissection and small enough for proper replication across multiple lab colonies.

Our study elaborates on a number of earlier insights in larval development of attine ants and contributes to an increasing trend to examine larval morphology (Wheeler & Wheeler 1976a, Fox & al. 2017) and the role that larvae play in superorganismal colonies (Schultner & al. 2014, 2017). Previous work suggested that attine worker development varies considerably from three to six instars (Hölldobler & Wilson 1990, Ramos Lacau & al. 2008). However, there are nearly 300 attine species (Branstetter & al. 2017) and studies often compared only final instars (e.g., Wheeler 1948, Schultz & Meier 1995), although some have examined changes in larval morphology during development (Torre-Grossa & al. 1982, Wheeler & Wheeler 1986). In a more recent study, head capsule width was used to distinguish larval morphs of *Cyphomyrmex transversus* Emery, 1894 suggesting five larval instars (Ramos Lacau & al. 2008). Larval morphology is also an underutilized taxonomic trait for differentiating between species, and larval traits have therefore been used in species descriptions and cladistic analyses of fungus-growing ants (e.g., Schultz & Meier 1995, Jesovnik & al. 2013, Sosa-Calvo & al. 2017). The earlier work which is of most direct relevance for our present study is the description of hair morphology and number of instars for gynes, males, and workers of *Acromyrmex octospinosus* (Reich, 1793) (Torre-Grossa & al. 1982). We therefore follow the terminology of these authors and operate under the assumption that workers and sexuals of *Acromyrmex echinatior* differ in the number of larval instars.

The overarching objective of our study was to conduct a comprehensive survey of live larval morphology of the fungus-growing ant *Acromyrmex echinatior* and provide clear documentation of larval development for males and the different female castes. Working with live larvae presented us with some limitations in what we could measure without risking the health of each larva. However, we hope that these time-limited methods will provide researchers with a quick guide to identifying larval castes. We had four aims: (1) identify the four larval instars of small and large workers; (2) identify the five larval instars in the gyne and male larvae; (3) discover key morphological characteristics that differentiate between castes and sexes across instars; and (4) provide an accessible morphological key that can be used on live specimens, both with a standard stereomicroscope and when having a z-stack system available.

## Materials and methods

**Biological material and rearing:** The eight colonies of *Acromyrmex echinatior* used in this study (Ae155, Ae360, Ae361, Ae376, Ae406, Ae420, Ae430, and Ae451) were collected in Gamboa, Panama, in 2001 (Ae155) and 2008 - 2010 and were kept in humidity (ca. 70%) and temperature (ca. 25 °C) controlled rearing chambers at the University of Copenhagen in Denmark. Species identity was confirmed using Schultz & al. (1998). Colonies were provisioned with bramble leaves and occasional portions of dry rice and apple and were thriving during the period of larvae sample collection (2014 - 2015). Vouchers were deposited at OSUC (Ohio State University, Columbus, Ohio, USA).

*Acromyrmex echinatior* subcolonies were reared so that the growth and development of larvae from the first to the final instar could be followed. The size of a larva can be used to estimate the time when a larva enters the next phase of development (Fox & al. 2017), which is important because growth trajectories influence the ultimate size and shape of ant larvae (Wheeler 1986). There are challenges for this type of work specific to fungus-growing ants, such as: (1) Fragments of the fungus garden can be peppered with young larvae that need to be removed or counted when left in; (2) garden fragments must be large enough to secure that workers assume and maintain normal activities of brood and garden care; (3) larval growth can slow down or even stop for several weeks due to unknown factors in the social colony environment (Dijkstra & Boomsma 2007, Dijkstra & al. 2010); and (4) if the ants do not actively provision the garden, it will dwindle in size, resulting in the death of brood and workers. Despite these challenges, a sufficient number of subcolonies with 10 - 15 young larvae each were maintained for up to six weeks, although observation of individual larval development was not always possible. Larvae known to have reached specific developmental stages were used for z-stacked photographs and Scanning Electron Microscopy (SEM).

**Z-stacking imaging methods:** Larvae were taken from mature lab colonies with a mother queen or from small queenless subcolonies. They were separated into a holding chamber, which was a closed Petri dish with a small fungus garden fragment, lined with moist cotton wool. Individuals were handled with wax coated, fine, soft forceps (BioQuip Catalog #4748, Rancho Dominguez, USA) and moved to a black paper square placed under a Leica MZ16 macroscope (Buffalo Grove, USA). An LED ring light and a dome diffuser were used (as in Kerr & al. 2008) so the focal specimen was not heated and remained alive. For specimens that were kept away from the holding chamber for longer periods of time, a small drop of water was placed on the black paper square to prevent desiccation while being photographed. All specimens were photographed live, which sometimes led to blurred images due to head movements. In these cases, the larva was placed in 70% EtOH for about 10 minutes to kill but not quite 'fix' the specimen. This allowed for life-like photographs without shrinking or whitening the tissue. The number of stacked photographs varied from five to 50 depending on the size of the specimen. Photographs were finally compiled with Zerene Stacker software (version 1.04 on PMAX setting) and saved as TIFF images.

**Specimen preparation:** Following the initial imaging of mostly live larvae, specimens were placed in a series of EtOH baths to slowly fix tissue without shrinkage. After an interval of $\geq$ 10 minutes, EtOH solutions of increasing concentrations (ca. 80%, 90%, and 100% EtOH) were added, and specimens were finally placed in an Eppendorf tube in pure 100% EtOH and stored in the refrigerator until they could be imaged with SEM or genotyped to determine whether they were female or male (see below).

If not prepared in EtOH as described above, live specimens were collected from colonies and killed when placed into about 96 °C water for 2 seconds and transferred immediately into 70% EtOH and dehydrated in a series of EtOH baths for 20 minutes at incremental concentrations of 70%, 80%, 90%, and 99.9%. Specimens were then dried using the Tousimis Autosamdri-815 critical point drier (Rockville, USA), mounted on aluminum stubs and coated with 80% platinum / 20% palladium using a JEOL JFC-23000HR (Peabody, USA), and then studied using a JEOL JSM-6335F scanning electron microscope at 7 kV and a working distance of about 36 mm.

**Genotyping methods:** Samples stored in EtOH were allowed to dry for about five minutes before DNA extraction. Larvae were cut in half on a clean glass plate. One half was stored in 96% EtOH and the other was added to 100 µl 10% Chelex 100 (Bio-Rad, Hercules, USA) in Tris with 5 µl proteinase K solution (Thermo Fisher Scientific, Waltham, USA). Samples were then centrifuged and incubated at 55 °C for 6 hours and boiled at 99 °C for 30 min. After 10 min, 50 µl of the supernatant were centrifuged. Genotypes of all samples were determined by amplifying DNA of eight highly polymorphic nuclear microsatellite loci (Tab. S1, as digital supplementary material to this article, at the journal's web pages), four of which were originally developed for *Acromyrmex insinuator* (Acrins 02, Acrins 05, Acrins 22, and Acrins 29) and four for *Acromyrmex echinatior* (Ech1390, Ech3385, Ech4126, and Ech4225; Ortius-Lechner & al. 2000, Hughes & Boomsma 2004).

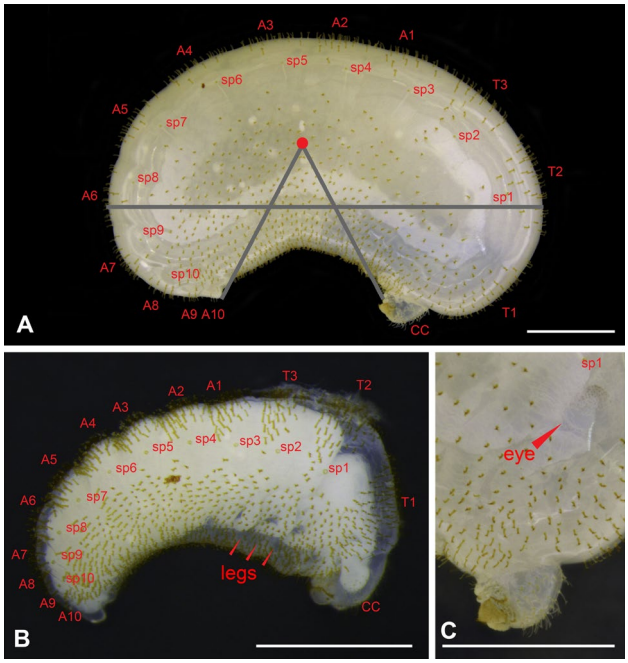



Fig. 1: Typical worker larval segmentation and morphological features. (A) Live large worker prepupa (4th instar) with translucent head and transparent "window" (ventrolateral thoracic region; T = thorax), where the legs are developing. Head capsule (CC = cephalic capsule) is empty. A few fat vesicles remain visible, but the gut (A = abdomen) is already cleared. Spiracles (sp) marks are positioned to the right of the sclerotized opening, and tracheae are faintly visible. The horizontal line illustrates the way in which length measurements were taken throughout this study, the inverted V illustrates the inner angle between mouth and anus, and the red dot is the center of the best-fitted ellipse around the larva. (B) Ethanol-preserved small worker prepupa (4th instar) with opaque legs (arrows). (C) Live large worker prepupa (4th instar) with translucent head, pigmentation of the eye just below the first visible spiracle (arrow), and abundant cephalic hairs. Background darkened for emphasis. All scale bars are 1 mm.

PCR reactions were performed in 10 µl volumes of 1 µl of DNA, 5.0 µl VWR Mastermix (Radnor, USA), dd$H_20$ and between 0.15 µl to 1.4 µl 10 mM forward / reverse primer. The PCR program began with an initial denaturation of five min at 94 °C, followed by 31 to 33 cycles of 30 s at 94 °C, 30 s at the locus-specific annealing temperature (Tab. S1), 30 s at 72 °C, and a final elongation of 20 min at 72 °C. For the Acrins primers, a touchdown program was used. Repeat lengths of microsatellite loci were determined on an Applied Biosystems, Hitachi 3130 xl, Genetic Analyzer (Waltham, USA) using Liz-500 as standard and HIDI formamid, after which banding patterns were analyzed using GeneMapper 4.0 software (Applied Biosystems).

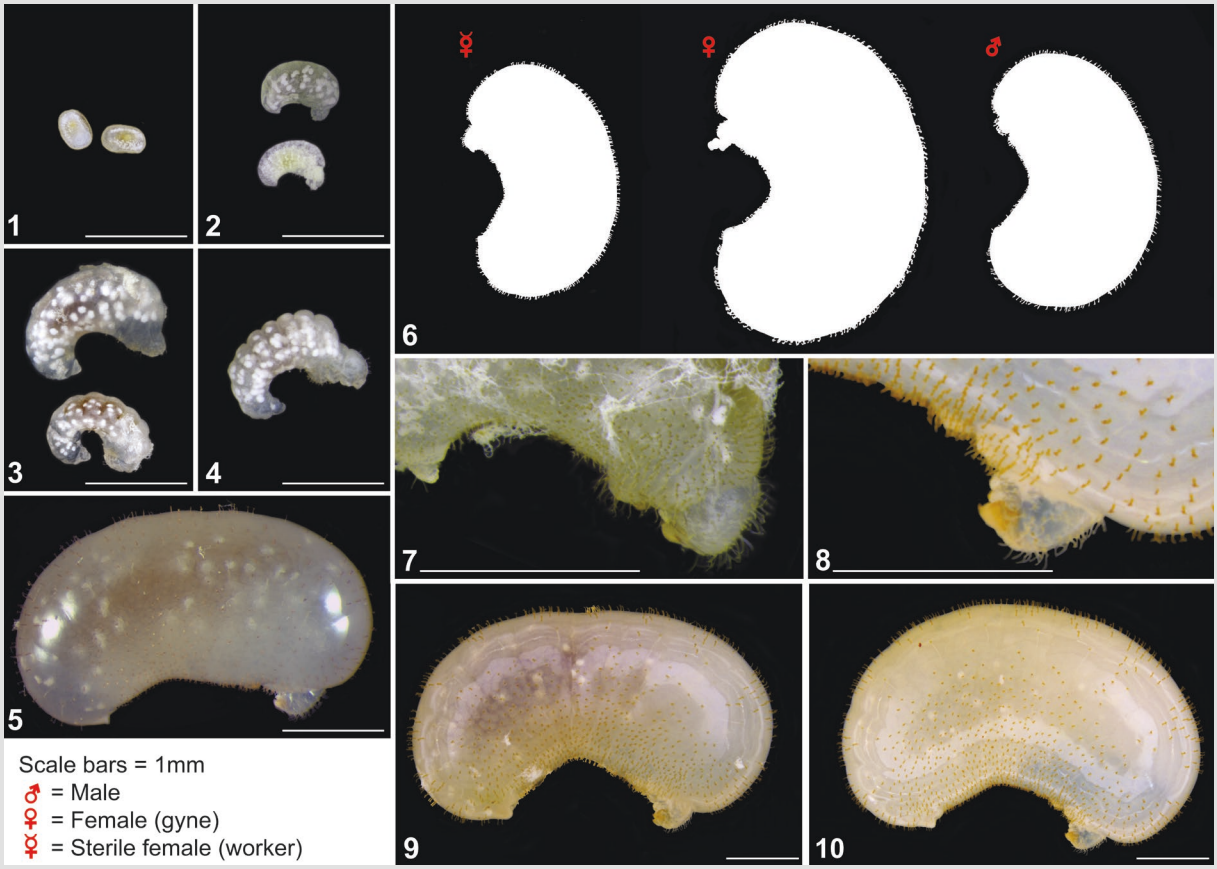
**Measuring z-stacked images, summary statistics, and terminology:** Using ImageJ, 234 individuals were measured (Rasband 2012). For each larval image, the length of the scale bar was recorded (for normalization) and then the positions of the tip of the mandible and anus. The whole body area was then selected and the center (pixel center of the selected area), aspect-ratio (major axis / minor axis of fitted ellipse), ferret (longest distance between any two pixels within highlighted area), position of the ferret, circularity (area * 4π / perimeter), and solidity were recorded (shaped area / concave area; see http://rsbweb.nih.gov/ij/docs/guide/146-30.html for details; Fig. 1A). The inner angle was calculated using the positions of mandible and anus relative to the center of the whole body and the outer angle was calculated using the relative position of the ferret to the center of body. The area of the head and body was also measured alone and then the head-body ratio for each individual was calculated (Fig. 1). Data analysis was done in R v4.0.2 (R Core team 2015) using two-tailed Wilcoxon rank sum test after visualization using ggplot2 (Wickham 2009, Vincent 2011), and relations between body length and developmental stage were evaluated by fitting a sigmoid growth function to the instar-specific scores along the first Principal Component axis (Fig. S1) based on body size, head size, head-body ratio, aspect ratio, ferret, circularity, solidity, inner angle, outer angle, visibility of legs (binary), and visibility of (filled) gut (binary; Fig. S1). To account for the differences among variables (e.g., continuous / binary, variance and skewness), the data were Box-Cox transformed and standardized using the caret package so that variables resemble standard normal distributions with means of 0 and standard deviations of 1 (Kuhn & al. 2015). The morphological terminology for larval pilosity and external morphology followed Torre-Grossa & al. (1982).

**Hair counts:** Hairs visible on the perimeter of larvae in lateral position (Fig. 1A - C) were counted for 104 z-stack imaged specimens (Tab. S2). Hair numbers are variable and not always symmetrical (Schultz & Meier 1995), but they are often used in ant larvae taxonomy studies, especially for the prepupal stage. The samples comprised of individuals from all instars, castes, and sexes, as identified by the key (Box 1) and by sometimes genotyping for ploidy across marker loci (male larvae are haploid and should thus appear as single band homozygotes for all marker loci, whereas females are diploid and should thus often appear as heterozygotes). In a few cases, head images were slightly blurred because the live specimen moved, but hair counting remained feasible. The dorsal (abdomen + thorax segments), ventral abdominal (A1 - 10 segments), ventral thoracic (T1 - 3), and cephalic (head) hair counts were recorded separately to obtain ratios of hair counts between the different body parts in the respective order. All statistics were performed in R v4.0.2 (R Core team 2015). A Wilcoxon rank-sum test was used to identify statistical differences between groups of larvae.

## Results

**Egg through second instar larva:** We were unable to differentiate sex or caste in the youngest individuals using morphology alone, although whole body area differed significantly between 1st and 2nd instar larvae ($p < 0.001$; Fig. 2A; Tab. S3). Eggs (0.408 - 0.442 mm length;

**Box 1**: A binary key for identifying the larval instars of the leaf-cutting ant *Acromyrmex echinatior*. All scale bars are 1 mm. The key is also available as an electronic application at <https://megalomyrmex.osu.edu/apps/acro-larva-key/>.




**1** Few or no body hairs (Figs. 1 - 4). Body length < 2 mm. ..... **2**

**1'** Entire body covered by many hairs (Fig. 5). Body length > 2 mm. ..... **5**

**2** Few to no body hair (Figs. 1, 2). Plump ovoid to crescentiform shape (Figs. 1, 2). Yellow egg yolk may be present (Figs. 1, 2). ..... **3**

**2'** Small aggregations of hair present on head (0 - 13), ventral thoracic region (1 - 22), or both (Figs. 3 - 4). Body is more elongate and C-shaped in dorsal profile (Figs. 3 - 4). ..... **(2nd instar) 4**

**3** No body hair (Fig. 1). Ovoid shape (Fig. 1). Yellow egg yolk present (Fig. 1). No protruding head (Fig. 1). ... **Egg**

**3'** Few to no (0 - 2) cephalic hairs present (Fig. 2). Few to no (0 - 7) hairs present on ventral thorax (T1 - 3; Fig. 2). Yellow yolk may be present (Fig. 2 bottom). Body is crescentiform in dorsal profile with protruding head (Fig. 2). ..... **1st instar**

**4** Ventral thoracic region swollen with obvious constriction anterior to head (T1; Fig. 4). Head protruding away from body and anus (Fig. 4). Many hairs (12 - 22) present on ventral thorax (T1 - 3; Fig. 4). Few to many (0 - 13) cephalic hairs present (Fig. 4). Ventral and dorsal abdominal surface (A1 - 10) without hairs (Fig. 4). ..... **Type II, 2nd instar**

**4'** Ventral thoracic region and head swollen with no obvious constriction anterior to head (T1; Fig. 3). Head sometimes pointed downward toward anus (Fig. 3 bottom). Few hairs (1 - 5) present on ventral thorax (T1 - 3; Fig. 3). Few (2 - 4) cephalic hairs present (Fig. 3). Few (0 - 2) hairs may be present on ventral abdominal surface (A1 - 10; Fig. 3). Dorsal abdominal surface (A1 - 10) without hairs (Fig. 3). ..... **Type I, 2nd instar**

**5** Body hairs mostly the curly type (Fig. 6 middle). Body shape may be of gyne type (thoracic segments and abdominal segments roughly equal in width; Fig. 6 middle). ..... **6**

**5'** Few or no curly type body hairs. Body shape not of gyne type (thoracic segments and abdominal segments may not be equal in width). ..... **8**

**6** Body length > 7 mm. Gut not visible (empty; Fig. 10). Head capsule transparent (head retracted; Fig. 8) and not moving. Developing legs visible (Fig. 10). Dark eye pigmentation visible within retracted head (just behind the head capsule; Fig. 10). ..... **5th instar gyne**

**6'** Body length < 8 mm. Gut visible (full, purple-brown color; Fig. 9). Head capsule is opaque (head present; Fig. 9) and may be moving. Developing legs not clearly visible. Eye pigmentation absent (Fig. 9). ..... **7**
**7** Body length = 5 - 8 mm. ..... **4th instar gyne**
**7'** Body length < 5 mm. ..... **3rd instar gyne**
**8** Hairs on ventral thoracic region mostly consist of Y-hair type (Fig. 7). Body shape of male type (thoracic segment width < abdominal segment width; Fig. 6 right). ..... **9**
**8'** Ventral thoracic region has no or few Y-shaped hairs; instead these hairs are more similar to hairs on the rest of the body and have an anchor-tip with a stout base (Fig. 8). Body shape not of male type (Fig. 6 left). ..... **11**
**9** Body length > 7 mm. Gut not visible (empty; Fig. 10). Head capsule is transparent (head retracted; Fig. 8) and not moving. Developing legs clearly visible (Fig. 10). Dark eye pigmentation visible within retracted head (just behind the head capsule; Fig. 10). ..... **5th instar male**
**9'** Body length < 8 mm. Gut visible (full; purple-brown color; Fig. 9). Head capsule is opaque (head present; Fig. 9) and may be moving. Developing legs not clearly visible (Fig. 9). Eye pigmentation absent (Fig. 9). ... **10**
**10** Body length = 5 - 8 mm. ..... **4th instar male**
**10'** Body length < 5 mm. ..... **3rd instar male**
**11** Gut not visible (empty; Fig. 10). Head capsule is transparent (head retracted; Fig. 8) and not moving. Developing legs clearly visible (Fig. 10). Dark eye pigmentation visible within retracted head (just behind the head capsule; Fig. 10)..... **(4th instar worker) 12**
**11'** Gut visible (full; purple-brown color; Fig. 9). Head capsule is opaque (head present; Fig. 9) and may be moving. Developing legs not clearly visible (Fig. 9). Eye pigmentation absent (Fig. 9). ..... **(3rd instar worker) 13**
**12** Body length > 4 mm. Head-body ratio is 0.01 - 0.02. ..... **4th instar large worker**
**12'** Body length < 4 mm. Head-body ratio is 0.01 - 0.06. ..... **4th instar small worker**
**13** Body length > 3 mm. Head-body ratio is 0.01 - 0.05. ..... **3rd instar large worker**
**13'** Body length < 3 mm. Head-body ratio is 0.04 - 0.07. ..... **3rd instar small worker**

0.268 - 0.292 mm width; n = 5) always had ovoid shapes and a transparent chorion and visible yellow yolk. They were not always easy to distinguish from 1st instar larvae (Fig. 2B bottom; Fig. 2C) because young larvae retained a yellow yolk-like coloration. However, eggs and 1st instar larvae clearly differed in body area and length ($p < 0.001$; Fig. 3A; Tab. S3). Other, presumably more mature 1st instar larvae had dark purple-brown guts (Fig. 2B top).

Eggs and young larvae were found in nursery aggregations in the top, middle, and bottom portions of the fungus garden (Fig. 2E), where larvae remained largely stationary, although they did wiggle from time to time. It seemed unlikely that the workers fed these tiny larvae individually, but they might have grazed upon the garden if positioned so that their mouthparts were near fungal gongylidia (Wheeler 1937). Like eggs and 1st instar larvae, 2nd instar larvae were still so small that they were difficult to detect in vivo against a natural fungus-garden background except when using a stereomicroscope (Fig. 2E).

The habitus of 2nd instar larvae often appeared flat and flexible when viewed in the garden (Fig. 2E), but their moderate to deep intersegmental constrictions were clearer than in 1st instar larvae (Fig. 2B, D), which were still plump and only slightly crescentiform in body shape. The overall C-shaped body and positioning of 2nd instar larvae in the garden suggested they had the ability to move independently. The purple-brown gut could be seen in translucent areas of the body between the fat vesicles of live individuals (Fig. 2B, D). Hairs appeared simple (i.e., without obvious bifurcations) when using a stereomicroscope.

We found two morphotypes (Type I and Type II) in the 2nd instar larvae that differed in whole body area ($p < 0.001$; Fig. 2A; Tab. S3) and body length ($p < 0.001$; Fig. 3A). They could be separated regardless of whether they were EtOH preserved or live specimens, primarily because they had notable hair patterning differences. Type I larvae had far fewer hairs (range 5 - 10; n = 12) along the perimeter of their bodies compared with Type II larvae (range 13 - 37; n = 17; Tab. S2).

**Third through fifth instar larvae:** Worker larvae. Both 3rd and 4th (prepupae) instar worker larvae were C-shaped in profile with similar hair patterning (i.e., dorsal lateral thoracic hairs with bifurcating anchor-tips) and segmentation barely visible as non-distinct constrictions (Fig. 4A, B). The beginning of leg development could be observed in 3rd instar workers, while developing legs were clearly visible in 4th instar workers (Fig. 1A, B). Developing eyes were prominent in 4th instar workers (Fig. 1C), while 3rd instar workers remained translucent, so the full guts and fat vesicles could be seen but not the eyes (Fig. 4A, B left). By the 4th instar, all worker larvae had cleared their guts, fewer fat vesicles were visible through the body wall, and larvae were never moving their heads or eating. The head capsule of 4th instar large workers had become empty and more translucent than opaque (Fig. 1A, C; Box 1).

At these more advanced stages worker larvae were bloated. They were often positioned in the garden with

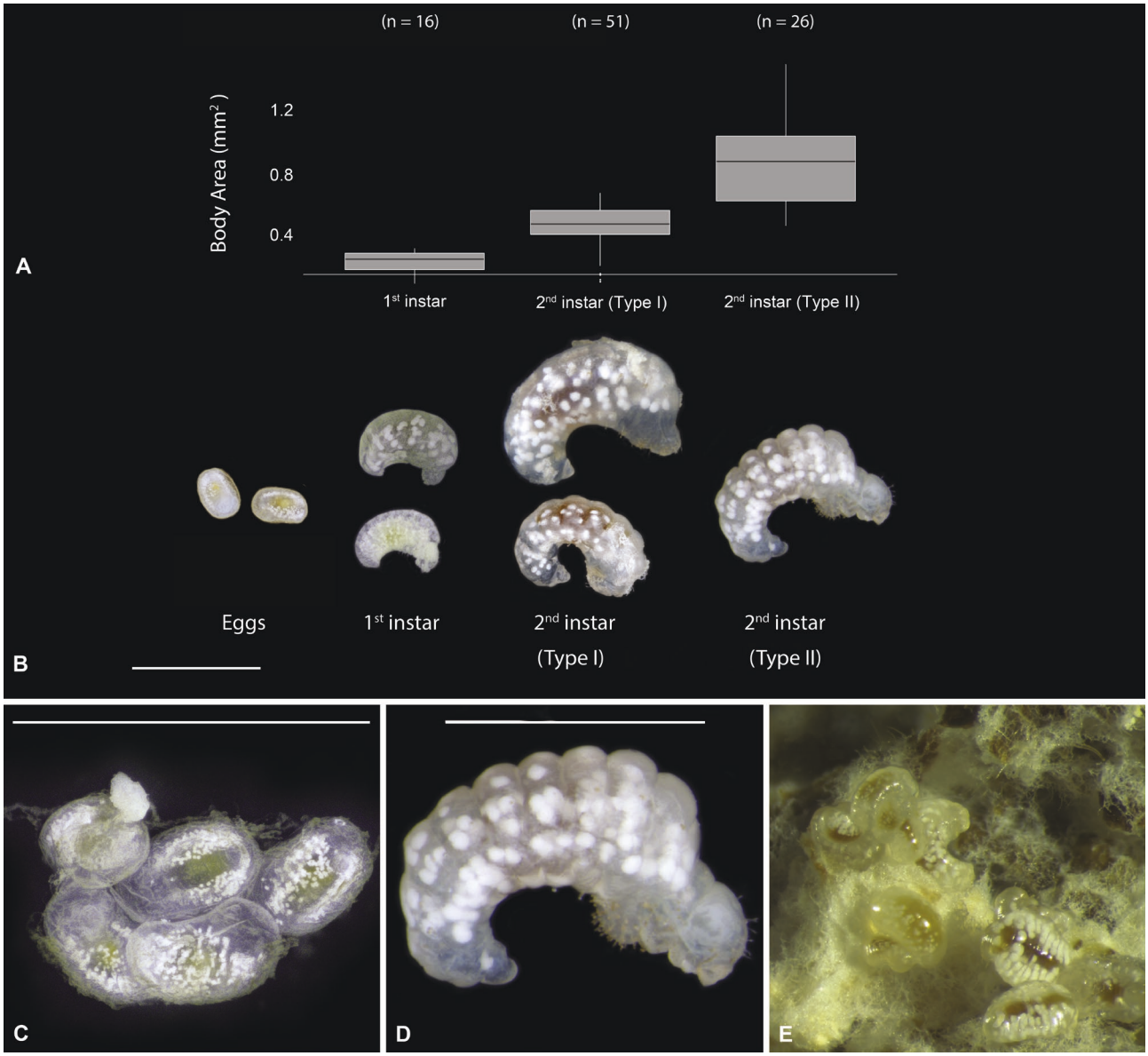



Fig. 2: The first developmental stages of *Acromyrmex echinatior*. (A) Box plot illustrating the body size difference between 1st and 2nd instar larvae of Type I and Type II (1st instar: mean = 0.27 mm², range = 0.15 - 0.36 mm²; 2nd instar Type I: mean = 0.48 mm², range = 0.16 - 0.68 mm²; 2nd instar Type II: mean = 0.96 mm², range = 0.49 - 1.44 mm²). Both stepwise increases were significant (two-tailed Wilcoxon rank sum test, $p < 0.001$). (B) Images of developmental stages from egg to 2nd instar, with the latter subdivided into Type I with a large head curved close to the posterior region with relatively few ventral thoracic hairs (both images were genotyped and determined to be female) and Type II with a swollen ventral thoracic region with dense hairs and the head orientated forward. The top Type I individual is an example of an intermediate specimen between the two types of 2nd instar larvae. (C) A cluster of preserved eggs found in the garden matrix and stuck together with fungal hyphae. Note the yellow yolk-like coloration that can also be seen in the bottom 1st instar larvae in the B-panel. (D) Enlarged 2nd instar Type II image from the B-panel to highlight the dense hairs on ventral thoracic surface (T1 - 3). (E) Z-stacked image of a garden "nursery" where hundreds of live young brood were located. Note the seemingly random orientation of the brood, indicating they may move about the garden by themselves to feed. Background darkened for emphasis. All scale bars are 1 mm.

their ventral side up and adult workers could be observed placing small bits of garden on the ventral thoracic region of feeding 3rd instar larvae. The head was positioned downward rather than forward, although this was more distinct in larvae of large workers than larvae of small workers (Fig. 4A, B). Tufts of fungus-garden mycelium could sometimes be observed on the body surface of smaller live individuals (Fig. 4A). Worker development was completed after four instars, and pupation followed soon after.

Male larvae. The main distinguishing characters between the males and workers were size (Fig. 3A), overall body plumpness (Fig. 4B, C), body shape (inner angle as in Tab. S3), abdominal segment width (Fig. 4A - C), and hair morphology (Figs. 5, 6C), although the size and body shape

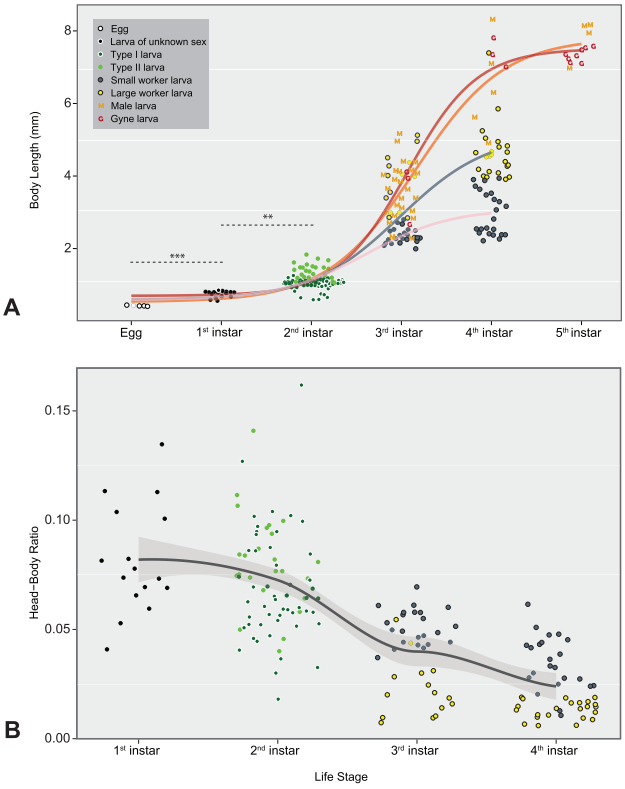



Fig. 3: (A) Body length of the life stages and castes of *Acromyrmex echinatior* reflecting growth and differentiation. Mean egg length is smaller than mean of 1st instar larval length (two-tailed Wilcoxon rank sum test, $p < 0.001$), which in turn is smaller than mean of 2nd instar larval length ($p < 0.05$). Larvae of gynes, large workers, and males in the 3rd and 4th instar are significantly larger than those that will produce small workers ($p < 0.01$). Body lengths of males and gynes are not significantly different in the 3rd instar, but 4th instar larvae of males and gynes are significantly larger than larvae that will become large and small workers ($p < 0.01$). (B) The ratio of head capsule area to body area predictably decreases as larvae develop. Sex of larvae remained unknown for the 1st and 2nd instars, but all others were workers. Head-body ratio was used (in addition to body length) to differentiate between the large and small workers in the 3rd and 4th instar as their hair morphology does not change. This measure was not needed for reproductives as there are no large and small castes to distinguish between. Type I and Type II larvae in the 2nd instar overlapped in head-body ratio but were significantly different from each other ($p < 0.02$). The head-body ratios differed significantly for larvae developing as large and small workers both in the 3rd ($p < 0.01$) and 4th instar ($p < 0.001$). Curves are fitted logistic regression models (Panel A) and local polynomial regression models (Panel B; Cleveland & al. 1992).

criteria only became unambiguously distinct in the 4th and 5th (prepupal) larval instar (Box 1). By the 4th instar, male larvae had generally longer bodies than larvae of large workers (Fig. 3A). However, there was some ambiguity in our samples and this underlines the importance of multiple lines of identification (e.g., body length and hair morphology) in our key. Male abdominal segments could also be more swollen than those of workers and of the male anterior thoracic region. All male larvae had deep bifurcating Y-hairs that stood erect with an angle of about 90 degrees between the two hair tips (Fig. 5 E - I), both on the dorsal and ventral mesothoracic regions and also on the lateral region. Leg development could be observed in 4th and particularly 5th instars, and developing eyes were sometimes visible. The bodies of 3rd and early 4th instar larvae remained translucent, allowing for visualization of full guts and fat vesicles (Fig. 4C). By the 5th instar, all male larvae had cleared their guts, there were zero to few fat vesicles left, and individuals were never moving their heads or eating. The head capsule of 5th instar larvae became empty and more translucent than opaque (Fig. 4C). Pupation took place following the 5th (prepupal) instar, and adult males emerged with larger gasters and proportionally smaller heads than workers.

Gyne larvae. Unlike any other larvae, gyne larvae had a distinct hair morphology, so that they could be unambiguously typed just based on hair-traits throughout the 3rd, 4th, and 5th (prepupal) instar. This is because gyne larvae had thick stout hairs with anchor-tips that appeared curled and visually close to the body surface (Fig. 6D). These hairs could easily be distinguished from the more erect Y-shaped hairs found on males (Fig. 6C) even when viewed just with a stereomicroscope (Fig. 5E; Box 1). The overall size and head-body ratios of male and gyne larvae overlapped, but this did not compromise identifications because gyne larvae had longer bodies than large worker larvae during the 4th instar (Figs. 3A, 4; Tab. S3). Leg and eye development could be observed in both the 4th and 5th instar. The body of 3rd and early 4th instar larvae remained translucent, so that full guts and fat vesicles could be seen easily (Fig. 4D), but all larvae had cleared their guts in the 5th instar and had few if any fat vesicles left. At this final prepupal stage, larvae were never moving their heads or eating, and the head capsule was also empty and translucent (Fig. 4D). Pupation took place following the 5th (prepupal) instar, and adult gynes emerged.

**Comparisons across castes and instars and trends in hair numbers versus hair shape:** Principal Component Analysis (PCA) indicated that about 80% of the total variation in five larval morphology variables could be captured by two axes (Fig. S1) and that PC2 mainly differentiated between the final (prepupal) instar and the earlier instars for which caste and sex were known. These data, and the assumption that workers represent a derived state compared with reproductives, strongly suggest that the 4th or 5th instar phase present in gynes and males was lost in worker larvae over time, as they require less development time. Through this putative loss, the 4th and 5th instars of workers and reproductives, respectively, are both prepupal stages. Another overall trend worth noting is that body pilosity steadily increased as larvae grew and molted (Tab. S2) but that those increases remained proportional across all body parts throughout development (Fig. S2). Combined with our extensive evidence that hair

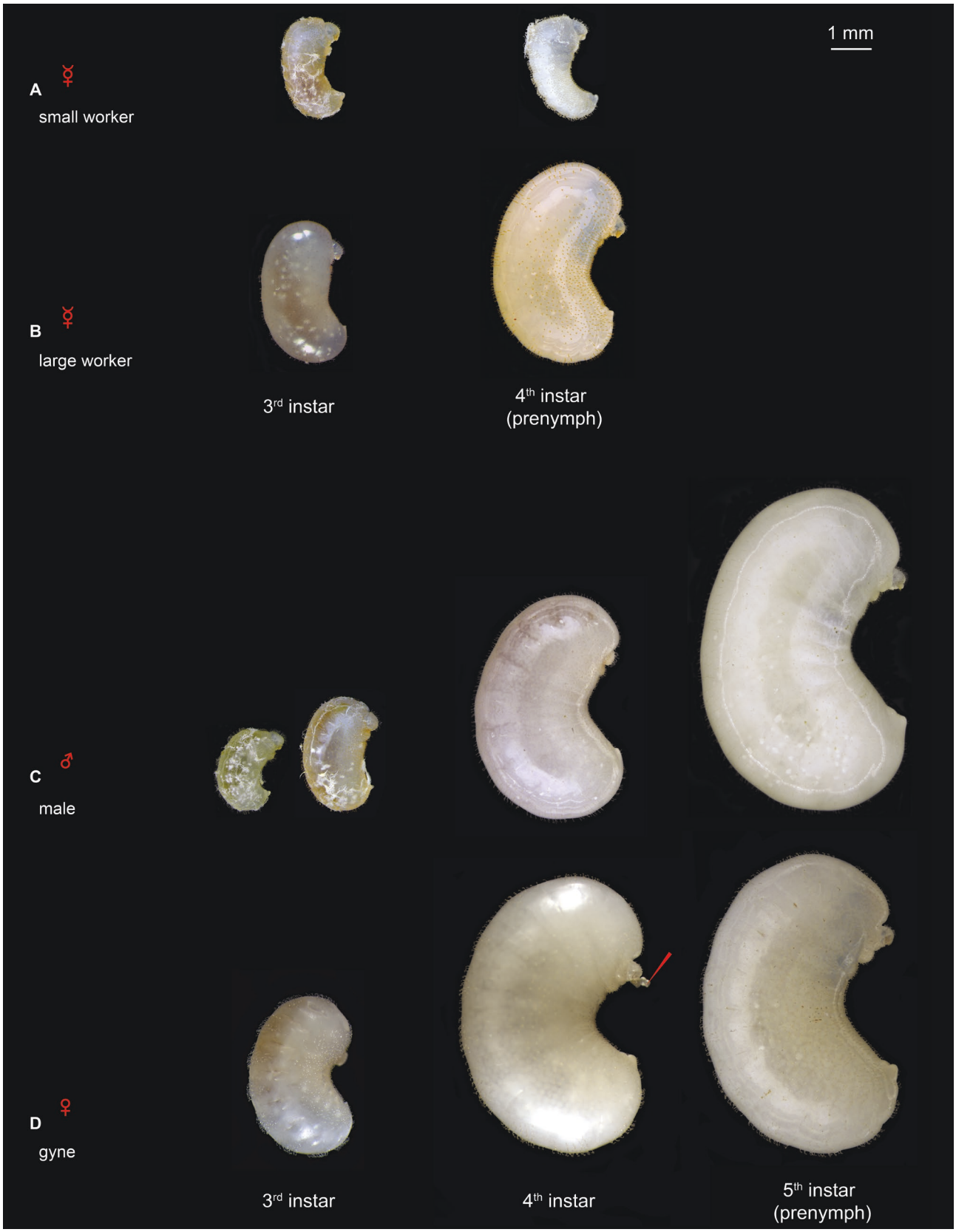



Fig. 4: Third and higher instars of live *Acromyrmex echinatior* larvae (lateral view). (A, B) Small and large workers (indicated by modified ♀) in the 3rd and 4th instar, respectively. (C) Two representative examples of 3rd instar male (♂) larvae, illustrating the range of body size and girth. The 4th instar individual has retracted its head and was moving when the image was made. (D) Female reproductives (gynes; ♀). Both 3rd and 4th instar larvae were moving when the images were made, and the 4th instar larva was feeding on a small bit of fungus (arrow). Background darkened for emphasis. All sized to the same 1 mm scale bar (numerical details in Tab. S1).

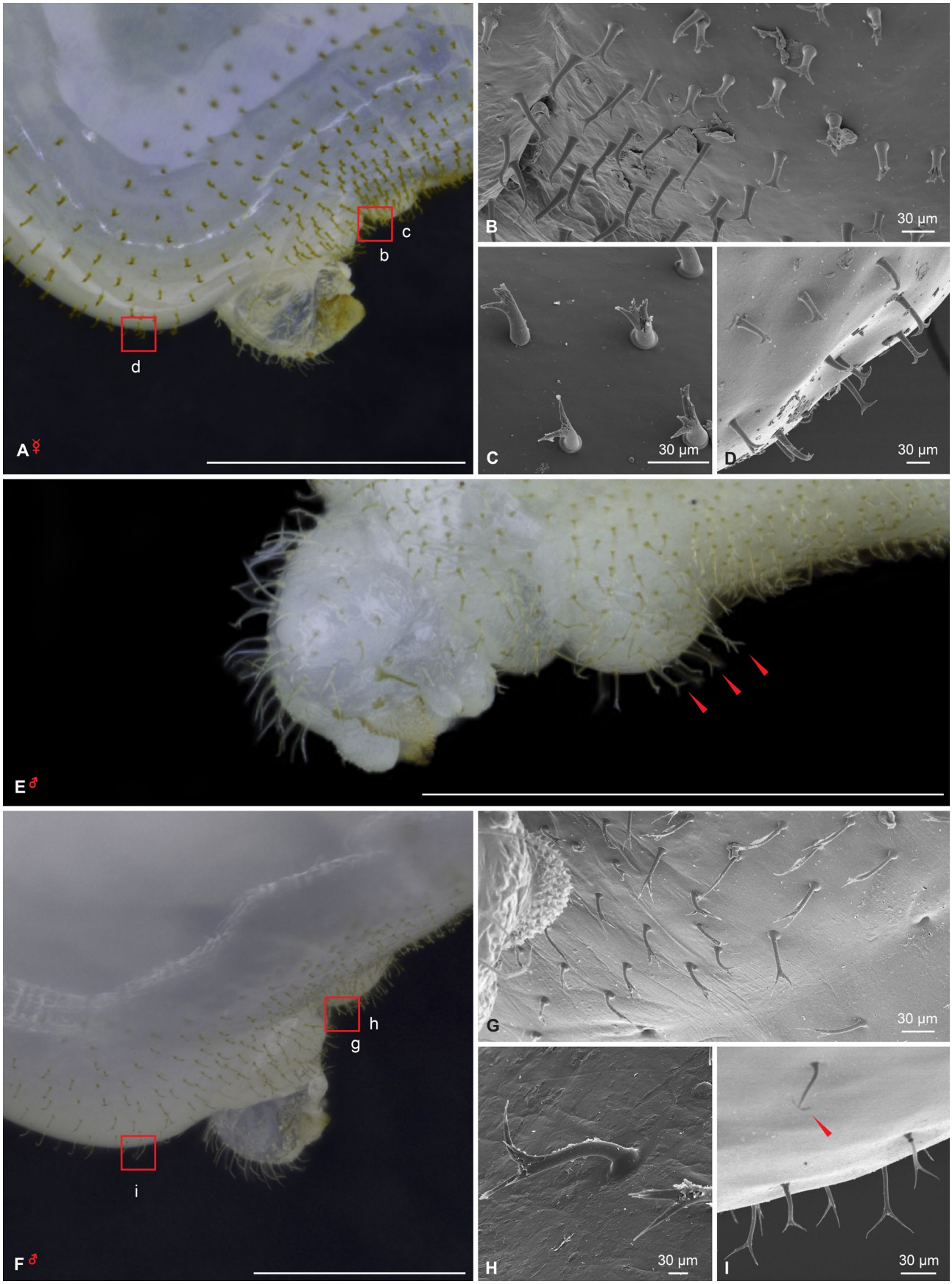



Fig. 5: Comparison of hair types in the thoracic regions of male (♂) and worker (indicated by modified ♀) larvae. Red boxes in z-stacked images (A, F) link to Scanning Electron Microscopy panels (b - d) and (g - i), respectively. (A) Lateral profile of a 4th instar (prepupal) worker larva. (B) Anchor-tip-like hairs from the ventral thoracic region of a 3rd instar worker larva,

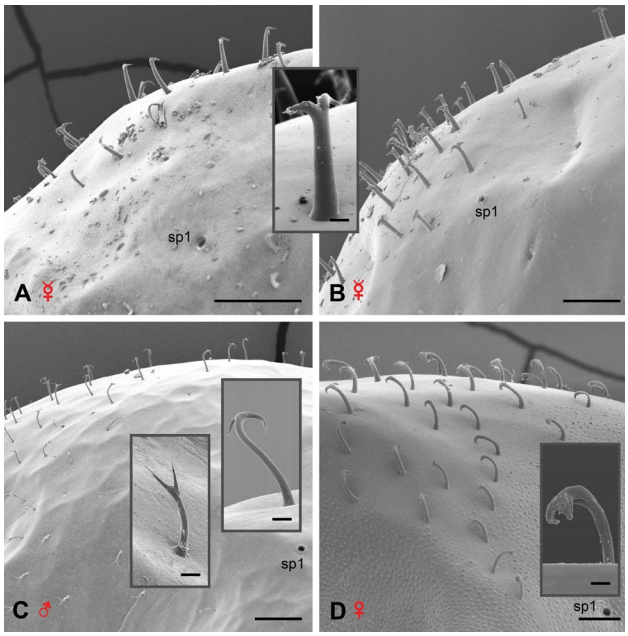



Fig. 6: Dorsal mesothoracic region of last larval stage, the first spiracle in view (sp1) for gynes (♀), males (♂), and workers (indicated by modified ♀). (A) Small worker (4th prepupal instar); (B) large worker (4th prepupal instar), insert: image of a typical short, stout, bifid, anchor-tipped hair found on the dorsal surface of all workers; (C) male (5th prepupal instar), left insert: example of the deeply bifid, Y-shaped hairs found on the lateral mesothoracic region, which are morphologically similar to the Y-shaped hairs found ventrally (Fig. 5 E - I); right insert: representative tall, slender, bifid, anchor-tipped hair found on the dorsal surface; (D) gyne (5th prepupal instar), insert: stout, bifid, anchor-tipped hair inclined towards body surface, making a curl-like appearance (Fig. 4D; Box 1). Note that anchor-tipped hairs branch with tips curling in opposite direction, while the Y-shaped hairs of males are straight and erect. All large images have 100 µm scale bars, and smaller insert images have 10 µm scale bars.

morphology became increasingly diagnostic for caste and sex (Figs. 5 - 7), these trends suggest that hair numbers may have general utility functions, such as helping larvae to increasingly move around independently to graze the fungus garden and that hair shape may be a social trait that allows nursing workers to continuously assess age, sex, and caste trajectory of the brood.

## Discussion

In this study, we set out to provide a comprehensive analysis of larval morphology for the fungus-growing ant *Acromyrmex echinatior* with the ambition to accomplish that goal with live individuals, using both a stereomicroscope to focus on traits important for field biologists and a standard z-stacking imaging system to obtain additional details. Despite various larval rearing challenges, we were able to accurately characterize the last two instars of small and large worker larvae and the last three instars of male and gyne larvae. However, we were unable to resolve the caste-trajectories of the first two instars. We did document that there are two distinct morphotypes of 2nd instar larvae, that can be separated by differences in the mean head-size versus body-size ratio (Fig. 3B). We also show that the sex of these small larvae can be determined using a set of microsatellite loci to assess whether individuals are haploid or diploid (only done for a subset of the specimens investigated). This simple genetic marker screening allows sexing of brood all the way back to the egg stage (Stürup & al. 2014). Overall comparisons further revealed that larvae gradually become hairier as they grow and molt but that the hair morphology is diagnostic for caste and sex, demonstrating that assessments become accurate from the 3rd instar onwards when enough hairs can be observed. PCA of a range of absolute and relative body size variables (Fig. S1) confirmed our diagnostics per instar and strongly suggested that the 4th larval instar of gyne and male reproductives is the penultimate instar. In combination, these traits allowed us to draw up a simple dichotomous key that we provide as an online resource (Box 1) and as a downloadable application <https://megalomyrmex.osu.edu/apps/acro-larva-key/>.

**Roadblocks towards a fuller understanding of leaf-cutting ant ontogeny:** Our study represents a significant diagnostic advance because most descriptive research on ant larval morphology has so far focused on a single instar, leaving the dynamic changes during development of individual ants unresolved (but see comprehensive list of studies in Solis & al. 2010). Torre-Grossa & al. (1982) concluded that pilosity and body length may be good for discriminating between larval instar in *Acromyrmex octospinosus*, but it takes considerable effort to convert these diagnostics into a binary key, as our study on the closely related *Acromyrmex echinatior* shows (Box 1). Only comparative data across instars can actually document what traits may have functional significance in social interactions and which characteristics may merely have general ergonomic significance. In contrast to the general inference by Torre-Grossa & al. (1982), our results suggest that overall pilosity is not important for social recognition but that hair morphology may be. The earlier *A. octospinosus* study noted that measuring the peritreme

showing variability in the extent of bifurcation. (C, D) Anchor-tipped hairs from the dorsal prothorax of a 3rd instar worker larva. (E) Y-shaped hairs on the swollen ventral mesothoracic region of an early 3rd instar male larva. (F) Lateral profile of a 5th (prepupal) instar male larva. (G) Ventral thoracic region of a 4th instar male larva. (H) Y-shaped hair from the ventral thoracic region of a 4th instar male larva. (I) Y-shaped hair from the dorsal prothorax (T1) of a 4th instar male larva (bottom) next to an anchor-tipped hair (arrow). Background darkened to improve contrast (A, E, F). All scale bars are 1 mm unless indicated otherwise.

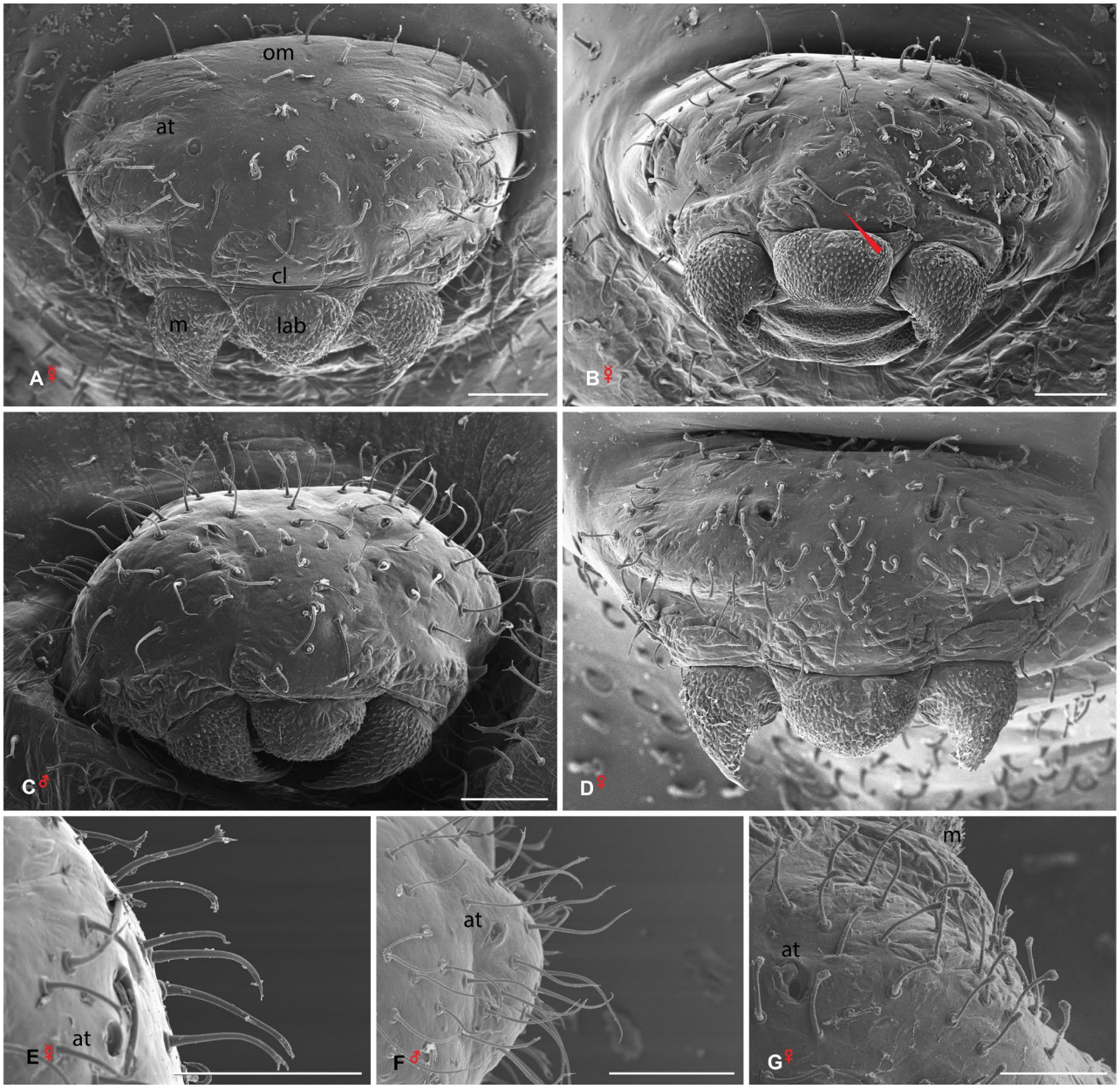



Fig. 7: Prepupa (final) instar for gynes (♀), males (♂), and workers (indicated by modified ♀). Head capsules in anterior view and pilosity patterning in lateral view. (A) Small worker (om = occipital margin, cl = clypeus, at = antenna, lab = labrum, m = mandible); (B) large worker with simple hairs located on the clypeus (arrow); (C) male; (D) gyne; (E) shallow, branched, denticulate, cephalic hairs of a worker; (F) simple, unbranched, cephalic hairs of a male; (G) bulbous, blunt-ended, simple hairs of a gyne. All scale bars are 1 mm.

diameter of the mesothoracic spiracle is an effective way to diagnose larval instars. However, that is a destructive method, which was later suggested to be replaceable by metric head-capsule data with little risk to the life of the larvae (Fox & al. 2017), a recommendation we followed in the present study. We thus obtained lateral head-capsule views that could be scaled against total body length in head-body length ratios, which provided substantial additional information (Fig. S1).

Cuticular hair morphology has been known to differ between workers and gynes across other ant species (Edwards 1991), which inspired us to obtain such measures both with simple and advanced microscopy. This turned out to be remarkably efficient for diagnosing all 3rd, 4th, and 5th instar larvae (i.e., all brood larger than ca. 2 - 2.5 mm in body length; Box 1). Gonopodial discs can be used to distinguish the sex of the largest larvae (Torre-Grossa & al. 1982), but we found that hair type and body shape were equally reliable indicators and easier to obtain, so we did not use gonopodial disc information in our key. However, the hair morphology was not an informative trait for the 2nd instar larvae. Our microsatellite marker data indicated that all 2nd instar larvae that we genotyped were female (Type I, n = 15; Type II, n = 14) because they

were diploid heterozygous at multiple marker loci. As they were sampled year-round and outside of the time period that lab colonies produce reproductives, they are unlikely to have had gyne developmental potential so that it seems most likely that these types exemplified incipient trajectories in small worker and large worker development. This would be consistent with head-body ratios being the only partial discriminator variable between the two types that we could identify. We are confident that a more extensive study focusing explicitly on first and second instar larvae should be able to resolve this issue.

**The social and ergonomic significance of hair type and hair number:** Ant colonies have been highlighted as both quintessential examples of harmonious superorganismal cooperation (Hölldobler & Wilson 2008) and as battlegrounds for potential reproductive conflicts regulated over evolutionary time (Ratnieks & Reeve 1992, Bourke & Franks 1995, Sundström & Boomsma 2001, Ratnieks & al. 2006, Helanterä & Ratnieks 2009). So far, studies of reproductive conflict have almost exclusively considered adult workers and queens as agents with sufficient power to pursue their own inclusive fitness interests by treating brood categories differentially according to relatedness (Edwards 1991, Bourke & Ratnieks 1999, Beekman & Ratnieks 2003). It was only recently that a synthetic review of the possibly active social roles of ant brood broadened this perspective (Schultner & al. 2017). Because ant larvae are not kept in cells like the brood of bees and wasps, it is reasonable to hypothesize that they may have some independent agency, particularly in the later instars, although the practical challenges of experimentally testing hypotheses on possible alternative strategies of ant brood remain significant. Several studies in *Acromyrmex echinatior* tested whether conflicts over sex ratio and male production via workers—rather than the multiply inseminated mother queen—could provide clues about the expression of reproductive conflict, but so far no evidence for split sex ratios or worker-son production in the presence of the queen has been found (Dijkstra & al. 2005, Dijkstra & Boomsma 2007, 2008). However, conflict over caste fate (Bourke & Ratnieks 1999) has been found to be expressed in at least one other study. This rare conflict materializes through cheating patrilines that have an increased representation in the number of gynes relative to the worker number (Hughes & Boomsma 2008), a phenomenon that has also been documented in honey bees (Withrow & Tarpy 2018). These studies, and others focusing on genetic aspects of worker caste polymorphism (Hughes & Boomsma 2007, Jaffé & al. 2007), have strongly suggested that social insects with multiply inseminated queens are likely to harbor rare mutants that selfishly bias caste fate by increasing the likelihood of developing as a gyne, but the proximate mechanisms mediating such cheating strategies have remained elusive.

The extensive variation in larval hair morphology suggests that workers may be able to detect the sexual identity of larvae, which would underline the hypothesis by Trivers & Hare (1976) that workers should be in full control over relative investments in gyne, male, and worker brood – especially, if the indirect worker fitness interests are in conflict with the mother-queen interests (Sundström & Boomsma 2001). This idea has been challenged by the sexual deception hypothesis (Nonacs & Carlin 1990), suggesting that when brood is at risk of being culled (typically male larvae), males would be under selection to hide markers of their sex in order to increase their chances of survival. In line with this, Pulliainen & al. (2021) provide some evidence that female larval castes do differ in surface chemistry, while this is not the case for male and female reproductives. Surface chemistry (i.e., cuticular hydrocarbons) in *Acromyrmex echinatior* and other leaf-cutting ant brood are rather muted (Richard & al. 2007), and differences between reproductive larvae have yet to be reported, thus strengthening our hypothesis that hair morphology could provide an alternative, tactile route for workers to use in larval sex recognition. Our results indicate that male and gyne brood of *A. echinatior* are similar in size in the 4$^{th}$ and 5$^{th}$ instars (Fig. 3A) but distinct in hair morphology (Fig. 6C vs 6D; Fig. 7F vs 7G). Likewise, male and worker size overlaps in the 3$^{rd}$ instar, but hair morphology is also distinct at this stage, albeit somewhat less. Furthermore, both males and workers have bifid anchor-tip hairs, but they differ in stoutness (Fig. 6A, B, insert vs 6C, right insert; also see differences in stoutness in Zolessi & González 1974, Wheeler & Wheeler 1976b, Penick & al. 2012). Hair morphology should therefore make sex and caste recognition of larval phenotypes transparent, possibly because selection for sexually deceptive male brood is weak because multiple queen-insemination implies that sex ratio optima for workers and queen hardly differ (Dijkstra & Boomsma 2008). Alternatively, Penick & al. (2012) describe similar anchor-tip hairs in *Pheidole rhea* larvae that function in larval adhesion to nest walls. A function like this may apply in *A. echinatior* larvae as well. Regardless, the sexual deception hypothesis does help make the generally important point that we need to better understand the roles of proximate recognition mechanisms within ant colonies. Combining routine microsatellite genotyping with assessments of larval morphology of the resolution obtained in the present study may therefore open fascinating new testbeds for studying the expression and regulation of reproductive conflicts, particularly if chemical cues emitted by the same ant larvae could also be measured (Schultner & Pulliainen 2020).

**Past and future perspectives:** As we highlighted in the introduction, the importance of studying ontogenetic development of colony-level phenotypes is becoming increasingly apparent. This idea was at the core of the original superorganism concept (Wheeler 1911), but it remained largely implicit because advanced microscopy and transcriptomic tools were unavailable or prohibitively expensive for large-scale use. These limitations are now being overcome in studies of gene regulation networks, chemical colony gestalt odors, and within-colony interactions (e.g., Stroeymeyt & al. 2018, Nagel & al. 2020,

Ulrich & al. 2020, Walsh & al. 2020). These approaches should soon become extendable to include phenotypic plasticity of individual ant larvae. This will allow us to answer questions such as: (1) Do larval morphologies change over the lifetime of perennial ant colonies (Oster & Wilson 1978) even though parental genes remain unchanged? (2) How comparable are evolution of development studies when examining colonial superorganisms and organismal metazoan bodies? This parallel was explicitly suggested by Wheeler (1911) as being likely but still awaits proper documentation (Boomsma & Gawne 2018). (3) If the superorganism analogy were accurate, would new studies reveal deeper clarity about the totipotency and pluripotency in organismal cells and superorganismal larvae? Specifically, how similar are traits related to signaling, modification of gene regulation networks, and susceptibility to complementary effects of the external and internal social environment? We believe that a firmer grasp of the morphology of individual ant larvae as pioneered in the present study may significantly contribute to a better understanding of the collective aspects of colony-level development.

Our study is one of the first (Bruder & Gupta 1972) encompassing surveys of quantitative and qualitative features of the morphology of ant larvae using z-stacking imaging, a technique that was instrumental in obtaining high-level resolution images. The results allowed us to identify most larval instars of the leaf-cutting ant *Acromyrmex echinatior*. It seems likely that comparable efforts will be worthwhile in other experimental ant model systems, of which rather few have been developed so far. Some ant models allow experimental control over the full life cycle of colonies, such as the clonal raider ant (*Cerapachys biroi*; see Oxley & al. 2014) and the pharaoh ant (*Monomorium pharaonis*; e.g., Nagel & al. 2020). The aforementioned species are parthenogenetic or inseminate queens that have lost ancestral mating and dispersal flights, which has greatly facilitated their 'domestication' as laboratory model systems. Another ant, *Cardiocondyla obscurior*, also became a well-studied model for understanding developmental polyphenism because its queen and worker life-history traits (e.g., small body size, fast generation time, and controlled mating) are also highly suitable for experimentation (Oettler & Schrempf 2016). Nevertheless, these same special characteristics also imply that other quintessential ant traits cannot be studied. The leaf-cutting ant *A. echinatior* has not been fully domesticated as a laboratory ant model because mating under laboratory conditions has not been feasible so far. However, its long-lived lab colonies offer many opportunities for studying social evolution in an ant lineage that has retained ancestral monogyny and queen-worker dimorphism while secondarily evolving uniquely derived traits such as multiple queen insemination and polymorphic worker castes. With larval morphology now being known in more detail, these ants will likely allow future work to understand social integration from a more ancestral developmental perspective.

**Acknowledgments**

We thank the Smithsonian Tropical Research Institute (Panama) for providing logistic help and facilities to work in Gamboa, the Autoridad Nacional del Ambiente y el Mar (ANAM) for permission to sample ants in Panama and to export them to Denmark, Sylvia Mathiasen for assistance in the molecular lab, David Nash and two anonymous reviewers for making unpublished microsatellite primers available.

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