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Original Article

Morphology, but not morphometry, identifies instars in *Ectatomma tuberculatum* ant

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Abstract

Holometabolous insects have evolved larvae distinct from adult forms, which is at the root of their ecological success and lifestyle diversity. Moreover, plasticity in larval development has yielded to different adult phenotypes in caste systems and sexual morphs, especially in ants. While understanding larval development and growth is crucial for many research questions, identification of larval instars remains challenging. Indeed, it has been recently suggested that studies based on size distribution alone have misjudged instar number in ant species. Here, we identified larval instars of *Ectatomma tuberculatum* (Olivier, 1792) by comparing the morphology and morphometry methods. We also searched for potential differences between female larvae that developed into reproductive or non-reproductive caste, that is, gynes or workers. Our results showed that chaetotaxy clearly differentiated four larval instars, whereas only three instars were separated by classical morphometric tools (i.e., size-frequency distribution, implementation of model, and test of Dyar's rule). This contradictory result comes from the partial overlapping of larval head sizes at first- and second-instar. Head growth rate was lower than expected at the first moult, and then not constant from instar to instar. Consequently, *E. tuberculatum* does not follow Dyar's rule. Furthermore, gyne larva size increased exponentially at the end of development, probably without adding instar. We confirmed that the first-instar larvae in ants could be inconspicuous and then easily ignored using morphometry alone. We hypothesized this early stage serves hatching and social functions. Overall, we recommend focusing initially on larval morphology and combining approaches in order to correctly identify larval instars in ants.

Key words: Development, growth, larvae, instar number, Hymenoptera, Formicidae, Ectatomminae.

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Introduction

How individuals develop has major fitness consequences at adult life-stage. In holometabolous insects such as ants, only larvae are able to grow (Hanna & al. 2023). Thus, the body size they reach prior to pupation determines adult body size (Nijhout 2003, Nijhout & Callier 2015) and potential reproductive success (Wiernasz & Cole 2003, Beukeboom 2018). Larval development is the result of complex interactions between physiological regulatory systems that depend on genotype, maternal effects, and both abiotic and biotic factors (Nijhout & al. 2014, Heming 2018). Variation in growth rate, instar duration, and instar number is likely to occur between and within species (Esperk & al. 2007a, 2007b, Tammaru & al. 2010). For example, the number of instars in ants ranges from three to six depending on species, sex, and caste (see tab. 1 in Solis & al. 2010a). Moreover, plasticity in development can generate different phenotypes from a single genome in response to environmental cues, yielding to polyphenism (Evans & Wheeler 2001, Nijhout 2003, Trible & Kronauer 2017, Yoon & al. 2023). Indeed, phenotypic plasticity in ants is associated with reproductive role in females (Wheeler 1986, Corona & al. 2016), worker polymorphism (Wheeler 1991, Alvarado & al. 2015, Wills & al. 2018), and variation in sexual morphs (Rüppell & Heinze 1999, Wolf & Seppä 2016, Oettler & al. 2019). All of these traits contribute to division of labour, which is considered to be the root of the impressive diversity and ecological success of eusocial insects (Wilson & Hölldobler 2005). However, to understand the proximate mechanisms of larval development and their functional implications at individual and colony levels, it is necessary to identify the larval instars, which is the first and not the least important step.

Two methods are mainly used to identify instars. They characterize larvae from different age cohorts rather than directly observing larval development and moults. The

first method is based on morphological differences, especially for body protuberances and cuticular processes, such as hairs, sensilla, and spinules (Wheeler & Wheeler 1976). These structures likely vary in size, shape, number, or distribution between instars and provide species-specific identification keys (e.g., Petralia & Vinson 1979, Baratte & al. 2005, Solis & al. 2010a, Adams & al. 2021). The second method relies on larva growth characteristics and measurement of sclerotized body parts, such as mandibles, spiracles, and cephalic capsule. The dimensions of sclerotized body parts only increase with moult, and the size increment is typically constant at each moult as formalized by Dyar's rule (Dyar 1890). Consequently, larval growth tends to follow a step-wise and regular (often exponential) progression from instar to instar. The number of larval instars could then be inferred from the size-frequency distribution, with each peak representing one instar. The peaks are identified by visual inspection and / or by implementing mathematical analyses or models (e.g., Jesus & Bueno 2007, Solis & al. 2007, 2009, Nondillo & al. 2011, Fox & al. 2017). However, when peaks are not clear-cut in the frequency distribution, Dyar's rule is used for testing instar numbers and avoiding an instar to be overlooked (Daly 1985). The test also serves to limit methodological errors due to inaccuracy in measurement and incomplete larval sampling. Possible instar numbers are tested by comparing measured size with those predicted by the rule, and calculated growth ratios with Dyar coefficient (e.g., Zara & Caetano 2001, Jesus & Bueno 2007). In holometabolous insects, the Dyar coefficient is expected to range from 1.3 to 2.2 (Dyar 1890, Cole 1980). In addition, the direct observation of larval development may be used for checking some specific instars (Baratte & al. 2005, Penick & al. 2012b, Masuko 2017).

Although rapid and successfully generalized (Berg & Merritt 2009), the morphometric method has been shown to be inaccurate in the Myrmicinae ant, *Strumigenys solifontis* (see Masuko 2017) and various insect taxa (Coleoptera: Kishi 1971, Allsopp & Adams 1979, Skuhrovec 2006, Morales-Ramos & al. 2015; Ephemeroptera: Fink 1982, 1984; Lepidoptera: Schmidt & al. 1977, McClellan & Logan 1994, García-Barros 2006, Calvo & Molina 2008; Plecoptera: Fink 1984; Trichoptera: Brittain & Bildeng 1995). Errors occur when adjacent instars overlap in size, when a particular instar is rare in the larval population, and / or when instar number varies within the species (Kishi 1971, Schmidt & al. 1977, Allsopp & Adams 1979, Fink 1982, 1984, McClellan & Logan 1994, Brittain & Bildeng 1995, Skuhrovec 2006, García-Barros 2006, Calvo & Molina 2008, Morales-Ramos & al. 2015). Masuko (2017) demonstrated that size-frequency distribution has neglected the first-instar larvae of *S. solifontis* because these larvae overlap in size with the second-instar larvae. Nevertheless, it is yet unknown whether this phenomenon is specific to *S. solifontis* or widespread among ants, and whether it could be unraveled by applying complementary morphometric tools.

To address these issues, we investigated larval instars of *Ectatomma tuberculatum* (Olivier, 1792). We tested whether morphology and morphometry are congruent methods to identify instar number in this species. *Ectatomma tuberculatum* is an Ectatomminae ant widely distributed in the Neotropics (Weber 1946, Kugler & Brown 1982). This species shows weak queen-worker dimorphism and no worker polymorphism (Hora & al. 2001, Fjerdingstad & Crozier 2006). Except during colony founding, variation in worker size between colonies is reduced (Dejean & Lachaud 1992, Hora & al. 2001). As adult body size results from larval development (Nijhout 2003), we expected that size classes of worker larvae would clearly be separated in *E. tuberculatum*. In addition, as genetic and environmental factors control growth and development in insects (Nijhout & al. 2014), we used colonies from the same population and reared the colonies under standard laboratory conditions (i.e., same food, constant temperature and hygrometry). Moreover, larval morphology has been well-documented in this species, but only in mature larvae (Wheeler & Wheeler 1952a, 1952b for a key determination of larvae including the genus *Ectatomma*), and larval instars have been described in only two species of the Ectatomminae subfamily: *Ectatomma edentatum* (see Antoniali-Junior & Giannotti 2001) and *Ectatomma vizottoi* (see Vieira & al. 2009).

Here, we compared instars identification using morphology and morphometry methods in *Ectatomma tuberculatum*. First, we characterized and categorized larvae on the basis of their external morphology. Second, we applied the morphometric tools (i.e., size-frequency distribution, model analysis, and testing Dyar's rule) classically used to discriminate instars to larval measurements. Third, we re-analysed measurement data after larvae had been assigned to their respective morphological class in order to examine whether larvae of the distinct morphological classes also differed in head size. Finally, we asked whether there are differences in morphology and size between female castes. Because of caste dimorphism, we expected that larvae developing into gynes (i.e., the future queens) would have different growth parameters than those developing into workers, or a supernumerary instar. Both morphological and morphometrical methods were applied to alive larvae, allowing us to systematically detect instar and use larvae in further behavioural protocols.

Material and methods

Ant colonies and rearing conditions

Stock colonies of *Ectatomma tuberculatum* were collected in two distinct sites in Apazapan (19° 19' 38" N, 96° 43' 21" W), Veracruz, Mexico in November 2011 and January 2016. The species was identified using Kugler & Brown (1982), and voucher specimens were deposited at Instituto de Ecología (INECOL, Xalapa, Mexico). Colonies were kept in the laboratory (T = 26 ± 2 °C, RH = 52 ± 8%,

light:dark cycle = 12:12 h) at the University Sorbonne Paris Nord (France). They were housed in plaster nests connected to plastic boxes where food and water were provided. They were fed twice a week with crickets, mealworms, and a honey-apple mixture.

In the laboratory, colonies produced workers throughout the year. Some of them also produced sexuals (i.e., females, males, or both) at certain periods of the year. Studied colonies were either stock colonies or colonies that derived from sexuals produced in the laboratory. Eight mature queenright colonies of two different types were selected. Four colonies were rearing only larvae that developed into workers ("worker larvae"), and four colonies were rearing larvae that developed into either workers or gynes ("worker plus gyne larvae"). Adult population of the colonies was recorded for several weeks before and after larvae were measured, in order to identify the type of colony. All studied colonies had a queen (except one colony that had two queens), 300 - 650 workers, and all the brood stages.

Morphology and morphometry of larvae

All immature individuals (i.e., eggs, larvae, cocoons) were removed from the colonies and placed in petri dishes with moist cotton balls. They were counted by stage observed under a stereomicroscope (M80, with integrated camera IC90E; Leica Camera AG, Wetzlar, Germany) and photographed with a micrometer slide for scaling. For larvae, whole body photographs were taken in ventral and side views, and head photographs were taken in anterior view using the appropriate magnification. Large larvae with retracted head were forced to stretch their head through thermal shock (4 - 5 min at 5 °C). In a few cases, larvae were moving too much or were curled up due to moulting, making photographs blurry and measurements impossible.

Larval instars were characterized on the basis of external morphology observed under a stereomicroscope. As moulting is mainly a change in the cuticle (Chapman 1998), variations in cuticular structures, especially body hairs, were observed in a sample of larvae sorted by size. The degree of sclerotization of the mouthparts was assessed by colour (Wheeler & Wheeler 1976). Larval measurements were obtained from the photographs using ImageJ software (version 1.53e, Schneider & al. 2012). The maximum width of the cephalic capsule was measured as it only increases during moult and typically defines insect instars (Dyar 1890, Daly 1985). Body size, on the other hand, increases continuously across instars as the larva feeds, and is used as a proxy for body mass. Body length was measured across the long axis of the body in ventral view (i.e., excluding the neck and the head). Body width was measured at its widest point (i.e., across the fourth or the fifth abdominal somite). In addition, a sample of eggs and cocoons were measured along the major and minor axes. As eggs, larvae, and cocoons are ellipsoidal or subcylindrical, their volume was estimated as an ellipsoid (Coleman 1991).

Data and statistical analyses

To determine whether morphology and morphometry provide consistent information to identify instars in *Ectatomma tuberculatum*, the morphological classes of the larvae were first described. From these data, the proportions of larvae per instar were calculated for each colony. Comparisons with theoretical distributions with equal proportions were conducted using Pearson's exact Chi-Squared test.

Second, head width data were analysed using the morphometric tools that are classically used to determine the number of larval instars. Head widths were represented as a frequency histogram to identify peaks and then instars. Peaks were detected by visual inspection and by implementing Kernel density analyses in order to estimate the probability density function of the dataset and then mathematically and objectively define the structure of the distribution (Silverman 1986). Density was estimated with Gaussian model, bandwith was selected using the rule of thumb, and confidence band was constructed with bootstrap debiased approach. The number of instars identified through morphometry (i.e., the peaks of the distribution) and morphology (i.e., the morphological classes previously observed) was tested using the Dyar's rule (see method in Floater 1996). Following this rule, head size is expected to increase at each moult with the same increment (i.e., Dyar's theoretical coefficient). Sizes predicted by Dyar' rule were calculated by dividing larval head widths by the average growth rate determined over the entire growth period (i.e., average actual size increment), from the last instar to the preceding one, and so on (Dyar 1890). Dyar's test supports the number of instars when there is an isometry between measured sizes and those predicted by Dyar's rule. To further analyse this point, head growth rates were calculated at each moult. These rates are defined as the mean size of an instar divided by the mean size of the preceding instar (Hutchinson & al. 1997). Growth rates were calculated from the larval age cohort of each colony studied (n = 8 colonies) and compared using Fisher-Pitman Permutation tests for independent samples, with colony as stratum. Growth rates were tested for fitting in the range of Dyar coefficient found in holometabolous insects (Jesus & Bueno 2007).

Third, after each larva had been assigned to its respective morphological instar, head width was analysed by running a generalized linear mixed model (GLMM) fitted with a gamma distribution. Larval instar was added as fixed factor, body length as a covariate, and colony identity as a random factor. In addition, early larva and egg volumes were compared to determine whether they differ. Due to difference in sample sizes, dimensions of all first-instar larvae (n = 31) were compared with a random, counterbalanced sample of eggs and second-instar larvae issued from the same colonies, using Fisher-Pitman Permutation tests for independent samples, with colony as stratum.

Finally, the morphology and dimensions of larvae were compared between the two colony types. Gyne larvae were identified based on the maximum values for body length

in the two colony types (Tab. S1, as digital supplementary material to this article, at the journal's web pages). Hair number was compared between worker and gyne larvae using Fisher-Pitman Permutation tests for independent samples. GLMM were run to compare head width and body length between colony types and between larval instars within and between colony type. In each model, larval instar, colony type, and their interaction were specified as fixed factors, head width or body length as a covariate, and colony identity as a random factor. Models were fitted with a gamma distribution. Larval growth curves were calculated for the two colony types and their slopes were compared (see Zar 1986). The dimensions of cocoons were compared between castes using Fisher-Pitman Permutation tests for independent samples, with colony as stratum.

All permutation tests were performed using the exact method or the Monte Carlo method with 1,000,000 permutations, and for multiple pairwise comparisons, p-values were adjusted using the Bonferroni-Holm method. For GLMM, post-hoc comparisons were performed using Tukey-Kramer multiple comparison adjustments to obtain corrected p-values. In all tests, statistical significance was set to 5%. Statistics were conducted using the packages Coin, MASS, and ks in R v.4.2.1 (R Core Team 2022) and proc GLIMMIX in SAS OnDemand (SAS Institute Inc., https://www.sas.com/).

Results

Morphology and determination of larval instars

The larvae of *Ectatomma tuberculatum* were pogonomyrmecoid: The thorax forms a thin neck, curled ventrally, and the abdomen was large and round at the end (Fig. S1).

Four morphological classes of larvae were identified and considered as instars (Fig. 1). First-instar larvae appeared hairless, unpigmented, and without distinct mandibles. They were usually mixed with the eggs in the colony, and some of them were observed hatching, confirming that they were first instar larvae. In contrast,

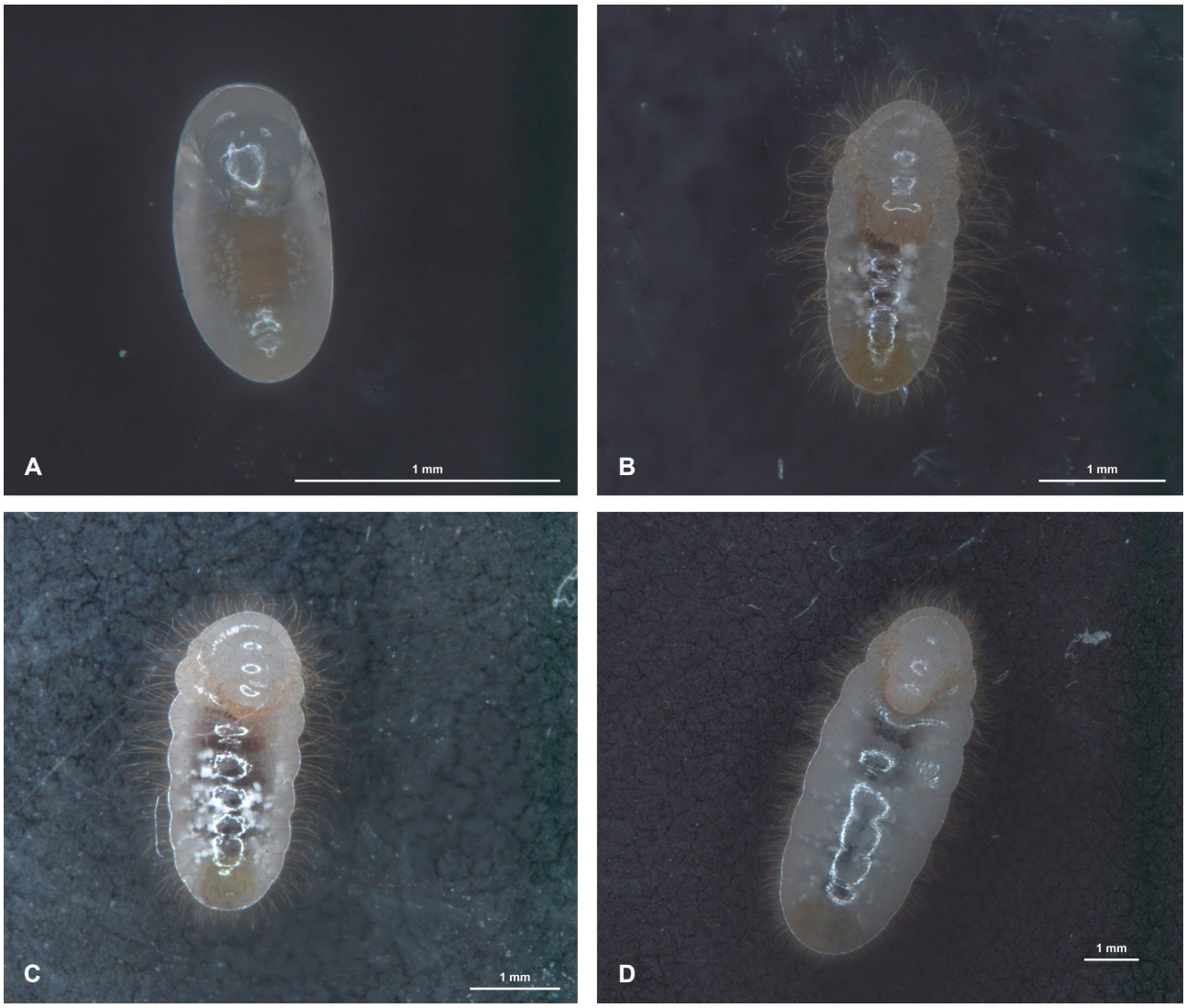

Fig. 1: Larvae of *Ectatomma tuberculatum*, ventral view. Worker larvae at instar I (A), II (B), III (C), and IV (D). The scale bar represents 1 mm.

Tab. 1: Number of hair rows on the first thoracic somite in *Ectatomma tuberculatum* larvae. Data are based on worker larvae (n = 15 - 31) and gyne larvae (n = 23) from eight colonies.

Instar	Larvae	Number of hair rows		n
		Median	Range	
I	Worker	0	0	31
II	Worker	2	1-3	15
III	Worker	4	4-5	15
IV	Worker	8	7-9	18
IV	Gyne	9	8-10	23

Tab. 2: Distribution of larvae in the four morphological classes, identified as instars in *Ectatomma tuberculatum*. Percentages are calculated from a total of 1005 larvae from eight colonies. Fourth instar included worker or worker and gyne larvae depending on the colonies.

Instar	Percentages of larvae (%)		n
	Median	Range	
I	4	2-6	34
II	20	16-29	218
III	32	23-41	310
IV	45	33-55	443

larvae of the subsequent instars were covered with body hairs and sparse head hairs. Hairs appeared unbranched, long, and flagelliform under stereomicroscope (terminology based on Wheeler & Wheeler 1976). Body hairs were arranged around somites in distinct rows with naked zones between somites. We observed that these hairs were organised in rows within each thoracic and abdominal somite, and the number of rows per somite was instar-specific (Tab. 1). Second-instar larvae had one to three hair rows per somite. Third-instar larvae had four or five hair rows, and fourth-instar larvae had more than six. Cephalic capsule was suboctagonal in anterior view and, except in first-instar larvae, appeared yellowish and then sclerotized. Head size appeared progressively small relative to body size across instars. In the three first instars, head was positioned ventrally on the abdomen and was entirely or partially visible. By contrast, when fourth-instar larvae were inactive, their head was completely retracted into the thoracic somites, and then not apparent. Mandibles were progressively larger and more sclerotized as larvae developed. In the last larval instar, the labrum and the apex of the mandibles were brownish, and thus presumably strongly sclerotized. Furthermore, when larvae were moulting from one instar to the next, the body was curved and appeared to be covered with abundant hairs and pieces of exuviae. At the end of development, larvae spun cocoons for pupation with the help of the workers.

Larvae of the four instars co-occurred in all studied colonies, but in different proportions. The first instar represented around 4% of the larvae, and the subsequent instars accounted for 20% to 45% in median (Tab. 2). These proportions deviated significantly from a uniform distribution for all the colonies (Pearson's exact Chi-Squared test, $p < 0.0003$).

Morphometry and determination of larval instars

Size-frequency distribution applied to head widths exhibited three major peaks with modes at 0.42, 0.51, and 0.63 mm (Fig. 2A), and Kernel density analysis identified three peaks, both suggesting three instars (Fig. 2B). As the number of instars differed between morphometry and morphology, we applied the Dyar's rule test to three instars (i.e., the three peaks of the size-frequency distribution) and four instars (i.e., the morphological classes), respectively. In both cases, the relationship between head width and instar apparently fitted the regression lines well and showed high coefficients of determination (Fig. S2). When considering three instars, measured and theoretical sizes predicted by Dyar's rule were aligned on a straight line with a 1:1 ratio (Fig. 3A). Growth rate was 1.24 ± 0.05 (mean ± standard deviation (SD), n = 8) for the entire larval development and was not significantly different at the first (1.26 ± 0.04) and the second moult (1.21 ± 0.04) (Permutation test, $p = 0.06$). These results suggest that the size increment is constant from instar to instar. Hence, Dyar's rule test agreed with three instars. However, when considering four instars (i.e., the four morphological classes), measured and predicted sizes deviated from a straight line (Fig. 3B). Average head growth rate was 1.19 ±

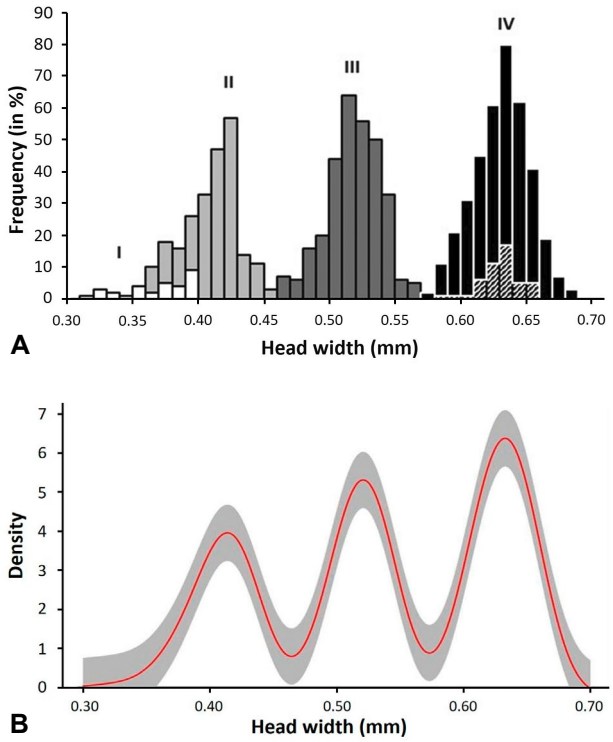

Fig. 2: Frequency distribution of head widths in *Ectatomma tuberculatum* larvae. (A) The distribution is based on pooled data from 936 larvae from eight colonies, after larvae had been assigned to their respective morphological class. Roman numerals indicate larval instars. The distribution of head widths partially overlaps between the first (in white) and second instars (in grey). Gyne larvae of the fourth instar are represented by hatched bars. (B) Kernel density estimates with 95% confidence band based on the same dataset as in (A).

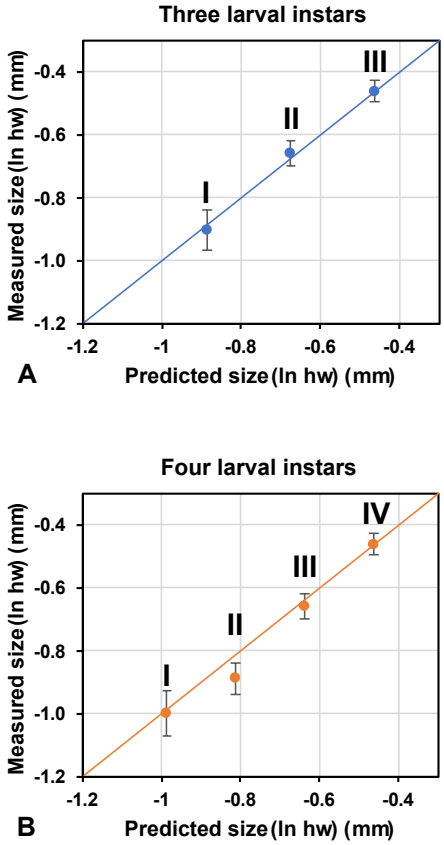

Fig. 3: Relationship between predicted and measured head widths when considering (A) three instars and (B) four instars, respectively. Head widths are log-transformed. Roman numerals indicate larval instars. Following Dyar's rule, points are expected to lie on a straight line with a ratio of 1:1. Measured head widths are mean ± standard deviation calculated from a total of 936 larvae (instar I: n = 31; instar II: n = 215; instar III: n = 307; instar IV: n = 383 larvae).

0.07 (n = 8) during the entire larval development, and more importantly, rates significantly differed between moults (Permutation test, $p < 0.0001$). Growth rate was significantly lower at the first moult (1.10 ± 0.04) than at second (1.26 ± 0.04) and third moults (1.21 ± 0.04) (Permutation test, $p = 0.02$ and $p = 0.02$), but did not differ between the second and third moults ($p = 0.06$). Hence, Dyar's rule test did not validate the four instars that are morphologically distinct, indicating that *Ectatomma tuberculatum* does not follow Dyar's rule.

After larvae were classified each into their morphological instars, we showed that distribution of head widths overlapped between the first two instars (Fig. 2A, Tab. S1). More specifically, 20 out of 31 first-instar larvae (64.5%) had head size within the range of second-instar larvae, and only 11 out of 31 first-instar larvae (35.5%) were smaller than the second-instar larvae. Head width was significantly different between instars (GLMM, $F_{3,929.3} = 2858.66$, $p < 0.0001$), and co-varied positively with body length ($\beta = 0.0023 \pm 0.0009$, $F_{1,932.4} = 6.22$, $p = 0.013$). Head width was significantly different between each consecutive instar, including the two first ($t \leq 15.92$, $p < 0.0001$, Tab. S1). In addition, we found that the three

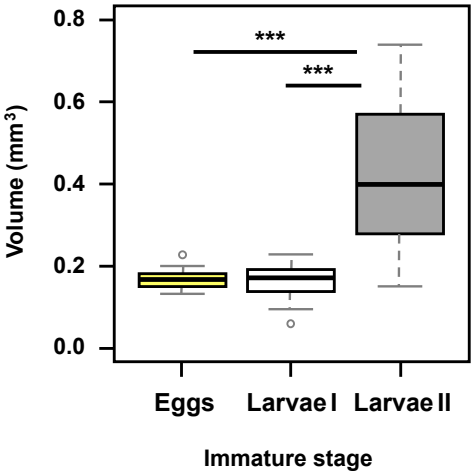

Fig. 4: Volume of eggs, first-instar larvae, and second-instar larvae in *Ectatomma tuberculatum* (n = 31 for each group). Boxes show median, quartiles, extreme values, and points show outliers. Permutation tests with Bonferroni-Holm correction, *** $p < 0.0001$.

early developmental stages differed in volume (Permutation test, $p < 0.0001$, Fig. 4), but only second-instar larvae were greater than eggs ($p < 0.0001$) and first-instar larvae ($p < 0.0001$). First-instar larvae did not significantly differ from egg volume ($p = 0.32$). Similar results were obtained when using length and width as dependent variables.

Specific features of gyne larvae

Gyne larvae were identified as over 8 mm long at the end of development (Tab. S1). Except body hairs that were more abundant in gyne larvae than in worker larvae (Permutation test, $p = 0.01$, Tab. 1), we observed no morphological differences between the two castes at any instar.

Larval head width significantly co-varied with body length and was significantly influenced by instar and the interaction between instar and colony type, but there was no single significant effect of colony type (Tab. 3). Head was significantly larger from instar to instar in both colony types ($t \geq 5$, $p < 0.0001$) and did not significantly differ between colony types for any instar ($t \leq 2.48$, $p \geq 0.21$). Moreover, the distribution of the head widths of gyne larvae completely superimposed that of fourth-instar larvae (Fig. 2A, Tab. S1). These results suggest that both female castes develop in four instars. Similarly, larval body length varied significantly with instar and the interaction between instar and colony type but did not differ significantly with colony type (Tab. 3). Body size increased significantly from instar to instar in both colony types ($t \geq 3.07$, $p \leq 0.045$). This increase was not significantly different in the two types of colonies ($t \leq 1.72$, $p \geq 0.67$), except for the fourth instar ($t = 4.67$, $p < 0.0001$). This indicates that gyne larvae growth is increased at the end of development compared with worker larvae. Consequently, slopes for larval growth curves diverged significantly in the two colony types ($t = 3.88$, $p < 0.001$, Fig. 5). As a result of this extra growth, gyne cocoons were greater than worker cocoons (Tab. S1; mean ± SD: 151.40 ± 17.60 mm^3 in volume, $n = 37$, for gyne cocoons, and 53.84 ± 9.70 mm^3 in volume, $n = 40$, for worker cocoons, respectively; Permutation test, $p < 0.0001$).

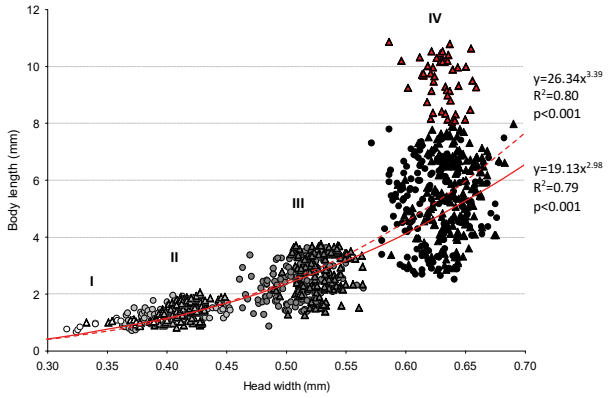

Fig. 5: Relationship between head width and body length in *Ectatomma tuberculatum* larvae, based on pooled data of 936 larvae from eight colonies. Roman numerals indicate larval instars. Circles represent larvae originating from the worker-producing colonies (n = 4 colonies). Triangles represent larvae originating from the worker- and gyne-producing colonies (n = 4 colonies). Triangles in red represent gyne larvae. Larval growth curves (in red), equations, and coefficients of determination are shown for the two types of colonies, that is, "worker larvae" (in solid line) and "worker plus gyne larvae" (in dashed line).

Discussion

Larvae of *Ectatomma tuberculatum* are differentiated in four morphological classes. These classes also differ in head size, and thus represent four distinct instars. However, morphometric tools identified only three instars that are wrongly confirmed using the Dyar's rule test. In contrast, four instars are not confirmed by the Dyar's rule test. Indeed, head growth is not constant throughout larval development, indicating that *E. tuberculatum* at least in the studied population, does not follow Dyar's rule. To our knowledge, deviations from Dyar's rule have not yet been demonstrated in ants. Consequently, morphology and morphometry methods are not consistent to identify instars in *E. tuberculatum*. Our results confirmed that size-frequency distribution is likely to underestimate the number of instars, as previously reported in the Myrmicinae ant, *Strumigenys solifontis* (see Masuko 2017). In both species, the method fails to discriminate between the first and second instars. This may result from larval growth pattern, as deviation from Dyar's rule shown in *E. tuberculatum*, and characteristic features of

Tab. 3: Results of linear mixed effects models analyzing the main effects of larval instar, colony type, and their interaction on the head width and the body length of larvae. Head size and length size are used as covariates. DF = degree of freedom; num = degree of freedom for numerator; den = degree of freedom for denominator; F = F-value; p = p-value.

	Head width			Body length		
	DF (num, den)	F	p	DF (num, den)	F	p
Larval instar	3, 929.1	2852.63	<0.0001	3, 915.7	29.37	<0.0001
Colony type	1, 8.8	4.20	0.0710	1, 13.5	4.03	0.065
Larval instar x colony type	3, 929.9	3.94	0.0083	3, 932.9	5.63	0.0008
Covariate	1, 929.9	9.33	0.0023	1, 847.1	27.82	<0.0001

first-instar larvae, that is, scarcity and small body size, that could make the instar elusive. These characteristics are likely to occur in other ant species, probably making the morphometric method not accurate enough as well. More specifically, previous studies on *Ectatomma* ants have distinguished three larval instars using size-frequency distribution (*E. edentatum,* see Antonialli-Junior & Giannotti 2001; *E. vizottoi*, Vieira & al. 2009). It is then unclear whether variation in instar number within the genus results from phylogenetic differences or from methodological issues. Thus, we suggest that morphometry should not be used alone to determine instars in ant larvae, and between the two methods, larval morphology should be favoured.

Chaetotaxy, that is, the arrangement of hairs and sensilla on any part of the exoskeleton, provides accurate and reliable morphological cues to discriminate larval instars in insects. In ants, the abundance and distribution of body hairs can vary from one instar to the next, and these changes are species-specific (Petralia & Vinson 1979, Wheeler & Wheeler 1986, Masuko 1990, Solis & al. 2007, 2009, 2010a, 2010b, Fox & al. 2012, 2017, Masuko 2017, Adams & al. 2021). In *Ectatomma tuberculatum,* larvae appear hairless at hatching and then become hairier at each moult. Body hairs are arranged in transverse rows, and the number of rows per somite increases with instars. This criterion refers to variation in hair number, a standard larval trait for identifying instars (Petralia & Vinson 1979, Wheeler & Wheeler 1986, Masuko 1990, Solis & al. 2007, 2009, 2010a, 2010b, Fox & al. 2012, 2017, Masuko 2017, Adams & al. 2021), and is very useful to sort living larvae under stereomicroscope. Hair morphology (i.e., type, shape, and length) can also characterize ant larvae. For example, in *Pheidole rhea,* the anchor-tipped hairs that serve to hang larvae to nest walls are typical of the last instar (Penick & al. 2012a). The types of hairs and their distribution on the different parts of the body (head, mandibles, thorax, abdomen) have been classified for each instar in several ant species (e.g., Petralia & Vinson 1979, Fox & al. 2007, Solis & al. 2009, 2010a, 2010b, Fox & al. 2012, 2017). In *E. tuberculatum,* larvae have unbranched hair type, but possibly due to limitation of our method, we did not find other hair types and differences between instars. This requires to be further explored using stereomicroscope and scanning electron microscope. Nevertheless, we identified four morphological classes of larvae in *E. tuberculatum,* and as larvae of these classes also differ in head size, they are considered as distinct instars.

Larval growth pattern shows a low head growth rate in *Ectatomma tuberculatum* (median: 1.21) compared with other holometabolous insects (median: 1.52, range: 1.3 - 2.2, Cole 1980), but appears in line with those in other ant species (e.g., Zara & Caetano 2001, Jesus & Bueno 2007). More importantly, *E. tuberculatum* does not follow Dyar's expectation that implies a constancy of size increment throughout larval development. To our knowledge, deviations from Dyar's rule have not yet been reported in ants. Variations in Dyar's coefficient between instars and between genetic strains have been demonstrated in other insect taxa, such as in *Manduca sexta* under nutrient laboratory restriction (Grunert & al. 2015). Even if some variations in larval development would be socially buffered in ants, our result is obtained on a single colony population and remains to be confirmed in other populations.

In the studied colonies of *Ectatomma tuberculatum*, the peculiarity of larval development explains why the size-frequency distribution fails to identify instars. Indeed, we found that head size of the second-instar larvae is smaller than predicted by Dyar's rule due to the limited head growth at the first moult. Physiological or mechanical properties of larval tegument could limit growth and impose constraints on developmental rule (Hutchinson & al. 1997). In *E. tuberculatum*, first-instar larvae have a reduced body size similar to the volume of the egg and, as we incidentally observed, moult 24 h - 48 h after hatching. We suggest that the first larval instar of *E. tuberculatum* would be a non-feeding stage and the subsequent instars would be growing stages. Non-feeding stages occur in basal orders of arthropods (Truman & Riddiford 2002). In ants, it may be inherited as an ancestral trait or derived secondarily from the evolution of sociality. Furthermore, reproductive division of labour in ants has decoupled brood production and brood rearing between queens and workers, respectively. Egg production depends mainly on queen fecundity and social structure, in particular the number of queens (Keller 1993, Purcell & Chapuisat 2012). As first-instar larvae can also characterize queens' investment in their offspring, their scarcity in the colony could be the result of at least two non-mutually exclusive reproductive traits, a low oviposition rate, and / or a small number of queens per colony. These two traits have been reported in *E. tuberculatum* (Hora & al. 2005, Fénéron & al. 2013) and *Strumigenys solifontis* (Masuko 2017, Wang & al. 2023), and could predict methodological difficulties in identifying larval instars.

Furthermore, the first larval instar of *Ectatomma tuberculatum* would have a hatching role. The absence of hairs on the cuticle is supposed to facilitate hatching (Masuko 2017) and is typical of this instar in many ant species (e.g., *Acromyrmex echinatior*, see Adams & al. 2021; *Eciton burchelli*, *Eciton hamatum*, see Wheeler & Wheeler 1986; *Odontomachus meinerti*, *Odontomachus bauri*, *Odontomachus brunneus*, see Fox & al. 2017; *Solenopsis invicta*, see Petralia & Vinson 1979; *Solenopsis helena*, see Fox & al. 2011; *Solenopsis saevissima*, see Fox & al. 2012; *Strumigenys solifontis*, see Masuko 2017). In addition, a specialised structure, that is, the egg burster, may have evolved to rupture the chorion during hatching (Pérez-de la Fuente & al. 2019). It should be found on the cuticle of newly-hatched larvae or remain attached to the egg chorion after hatching. Even if no sclerotized body part was detected under stereomicroscope, ultrastructural analyses of *E. tuberculatum* larvae are still needed. Alternatively, but not exclusively, the first larval instar could have a social function. For example, these larvae

could signal egg hatching to the workers and stimulate their transport to the larva pile where they will be fed. As body hairs usually serve as larval clumping (Wang & al. 2017), the hairless cuticle could prevent eggs from attaching to it and facilitate larva transport one by one as they hatch. Brood sorting is common in ants and is assumed improving brood care, adjusting developmental stages to local abiotic conditions and maximizing brood retrieval when changing nest sites (Hölldobler & Wilson 1990, Franks & Sendova-Franks 1992). The fact that eggs and early larvae are chemically distinct and discriminated by worker ants (de Fouchier & al. 2023) could be used as a proximate mechanism supporting this hypothesis. More generally, brood recognition relies on a variety of cues, including hairiness, which can differentiate developmental stages, as well as caste, sex, and species (Schultner & Pulliainen 2020).

Except for body size, morphology generally distinguishes female castes only in mature larvae (Wheeler & Wheeler 1976, but see Edwards 1991, Adams & al. 2021). These differences vary from weak to pronounced, depending on the species (Wheeler & Wheeler 1976, but see Edwards 1991, Adams & al. 2021). In *Ectatomma tuberculatum*, gyne larvae are hairier than worker larvae, but the variation is too limited to be used for caste discrimination. It is necessary to find additional clues, such as hair types, for example using the method successfully applied to *Acromyrmex echinatior* (see Adams & al. 2021). Queen development in *E. tuberculatum* appears to result from an exponential growth at the final instar, without adding instar. However, it remains unknown at which instar queens differentiate. As Penick & al. (2012b) suggested, queen differentiation can occur late during larval development in ants with weak caste dimorphism, such as *E. tuberculatum*. This confirms that it is necessary to identify characteristics specific to gyne larvae.

In conclusion, we confirmed and generalized by using different tools the fact that morphometry does not always result in appropriate identification of larval instars in ants. This issue has now been demonstrated in two species from phylogenetically distinct subfamilies. Thus, some caution is required when using the morphometric method, particularly in the context of interspecific comparisons or when studying polymorphic ant species. The first instar could be cryptic because of larval growth rate, reduced body size, low queen fecundity, and / or small number of queens per colony. Some of these biological parameters could be associated with possible methodological difficulties to identify instars. From a practical point of view, we suggest (1) collecting and rearing ant colonies in the laboratory to get all larval instars, (2) sampling individuals of all immature stages to sort young larvae from eggs, (3) isolating eggs from the colony to check their hatching in order to identify the first instar unambiguously. Overall, we recommend first focusing on larval morphology and combining approaches in order to determine larval instars in ants.

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Declaration on use of generative artificial intelligence tools

The authors declare that they did not utilize generative artificial intelligence tools in any part of the composition of this manuscript.

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