

# Morphological variations in the pre-imaginal development of the ponerine ant *Diacamma ceylonense*

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## Introduction

In ants, key developmental choices are made during the pre-imaginal phase. Although many studies precisely describe larvae of a large number of species for taxonomic purpose (Wheeler 1910; Wheeler and Wheeler 1979), an account of the complete sequence of larval development, from hatching to nymph, is often lacking. Vague terms, such as 'very young larva', 'immature larva', 'submature larva', 'mature larva' and even 'adult larva', can be found in literature (Wheeler and Wheeler 1952, 1989) and developmental studies are generally restricted to the biggest larvae, which unwittingly introduces a bias in our understanding of developmental processes. Only recently, a few studies have described the number and duration of larval instars in some species (reviewed in Hölldobler and Wilson 1990; Masuko 1990).

The larvae of Formicidae (Apocrita suborder of Hymenoptera) are grub-like and legless, in contrast to the caterpillar-like larvae of the Symphita suborder. Apocritous larvae from all species, both solitary and eusocial, are dependent on adults in most life functions: nutrition, cleaning and defence. In ants, brood care has reached a particularly high degree of complexity: nursing workers are able to recognize developmental stages of the brood and to transport them to different

## Abstract

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In the present paper, the larval development of the queenless ponerine ant *Diacamma ceylonense* is studied. Four instars were identified on the basis of cuticular processes – tubercles and spinules – which show discontinuous variation during growth and provide precise and reliable external morphological criteria for instar discrimination. In the first three instars, the larva possesses a striking proboscis, which disappears between the third and fourth instars. Larval weight increased around 50-fold in the 21 days of larval life between eclosion and pupation (mean weight for first instar = 0.37 mg; for fourth instar = 20 mg). In addition, the morphology of the gemmae, structures resembling vestigial wings in workers, is described in nymphae.

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parts of the nest that exhibit variations of temperature and moisture. Larvae are often assisted by workers in moulting and cocoon spinning (Wheeler 1910). As a consequence, a larva cannot be isolated without dying and cannot be manipulated or individually marked without being rejected by the workers.

Ants of the *Diacamma* genus belong to the phylogenetically basal subfamily Ponerinae. They are about 1 cm long and live in the tropics from south-east Asia to Australia. They present several interesting features for developmental and behavioural studies. First, as in a very small number of other 'queenless' ants (Peeters 1993), the queen caste has been lost in this genus. Each sex is represented by only one morph: winged males and apterous female workers. Reproductive division of labour is preserved: in each colony, a single worker, termed 'gamergate', is able to mate and lay eggs. Second, when *Diacamma* workers emerge, they possess a unique structure among ants: a pair of tiny dorsal mesothoracic appendages termed 'gemmae' (Tulloch 1934; Peeters and Billen 1991). Their gemmae are systematically bitten off by the gamergate whose own gemmae stay intact. Mutilation leads to a drastic and irreversible change of behaviour in the mutilated worker: it is no longer able to mate (Peeters and Higashi 1989; Fukumoto *et al.* 1989). The position of the

gemmae is similar to that of the male's forewings (Bitsch and Peeters 1991), suggesting that these structures are homologous. This hypothesis is supported by the fact that the nervous arborizations of the two structures are similar (Gronenberg and Peeters 1993). However, unlike wings, gemmae are filled with exocrine cells (Peeters and Billen 1991), the secretion product of which remains unknown.

The present work is aimed at describing the postembryonic development of *Diacamma ceylonense*. We chose to base our determination not on size and/or weight characters but on more reliable cuticular characters (following previous studies Delage-Darchen 1972; Petralia and Vinson 1979; Masuko 1990). We identify four distinct instars on the basis of their cuticular processes. Knowledge of this developmental sequence will help in describing the development of gemmae and how they may differ from wings in further studies.

## Materials and Methods

### *Ant colonies*

Colonies of *D. ceylonense* were collected in Bangalore, Karnataka State (India) and housed in plaster of Paris nests into which chambers had been moulded. A removable glass plate covered each chamber, allowing individuals to be taken out. Colonies were maintained at 25 °C under a 12-h light : 12-h dark photoperiod and regularly provided with a diet of *Tenebrio* larvae and nymphs, honey and distilled water. Relative humidity in nests was maintained at high levels by regularly moistening the plaster.

### *Collection of larvae and pupae*

To measure growth, larvae of different sizes were collected and weighed. Since larvae are soft, curved and distinctly segmented, length cannot be easily measured and is not a reliable measure of growth. For pupa collection, cocoons were removed and opened. Male brood was obtained from 'orphaned' colonies, where the gamergate was absent and where unmated workers laid haploid, male eggs (Peeters and Tsuji 1993).

### *External morphology*

For scanning electron microscopy (SEM), specimens were fixed for 30 min in phosphate-buffered saline with 4% paraformaldehyde at room temperature and gradually immersed in 100% methanol. After dehydration, specimens were coated with gold-palladium and viewed with a JEOL JSM 840 A electron microscope.

### *Estimation of instar duration*

The duration of each instar cannot be directly determined on the same individual because manipulation of larvae always

leads to their rejection by workers and to their death. Therefore once instars could be confidently characterized on the basis of morphological criteria, the number of larvae of each instar was monitored daily in small colonies with few larvae (from 2 to 6). When the number of larvae of a given instar changed between two observations, it was assumed that this was the result of moulting from one instar to another. When the number of larvae of a given instar decreased while the number of individuals in the subsequent developmental stage remained constant, it was assumed that some larvae died. This simple approach made it possible to estimate the duration of each instar without labelling individual larvae.

## Results

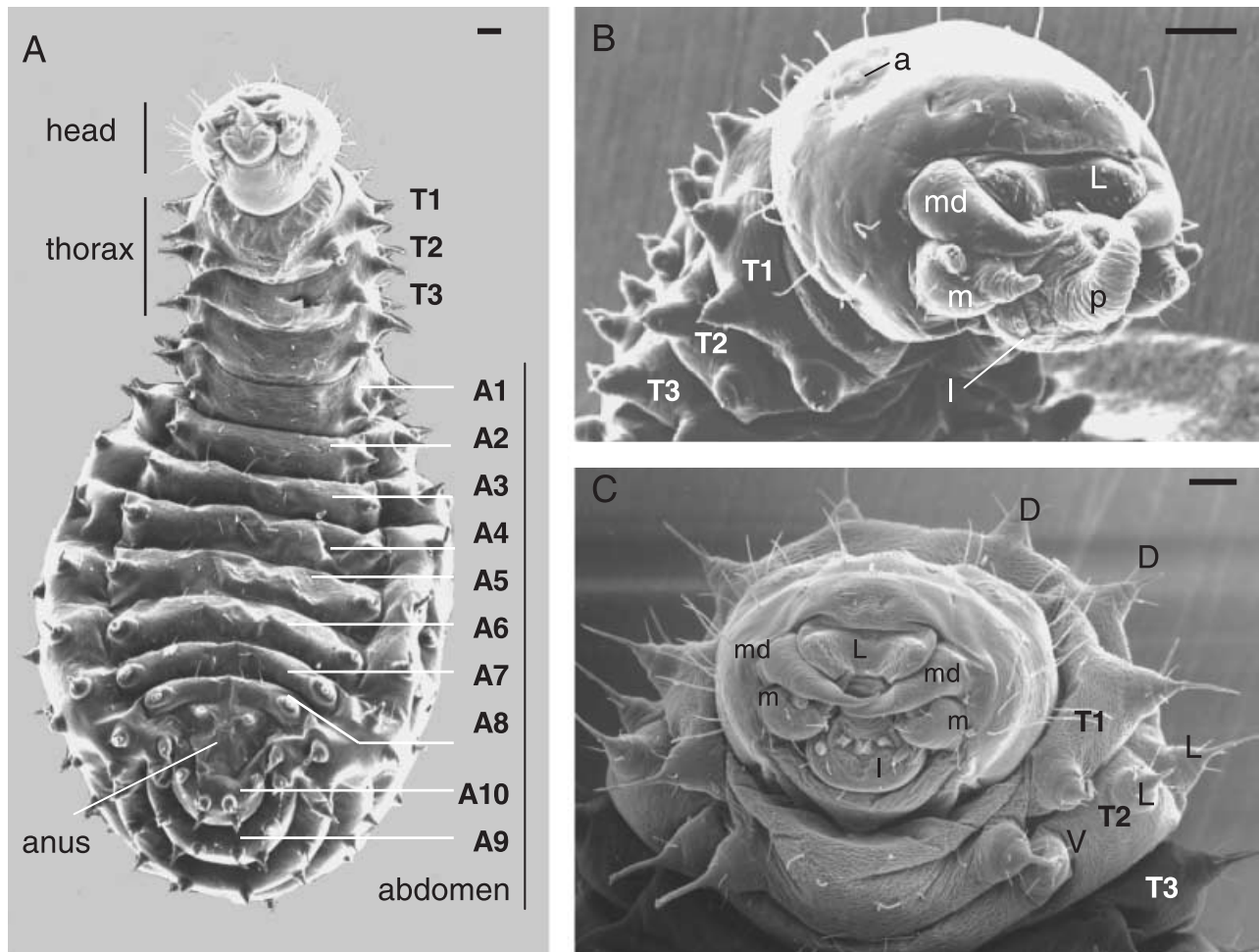
### *External morphology*

As in most ponerine ants, *Diacamma* larvae exhibit a small but distinct head. Sensory structures are quite reduced and scarce: few sensory hairs, minute antennae and no trace of eyes. However, the mouth parts are complete: a labrum, a pair of mandibles, a pair of maxillae and a labium, which is prolonged by a distinct and median proboscis (Figs 1A,B and 2A). The latter exhibits no apical opening and no spinule (Fig. 2B) but has long and multibranched hairs on its distal extremity (Fig. 2C) that may be involved in chemoreception. The mandibles and maxillary and labial palps are brownish and clearly sclerotized. Since ponerine ants rarely engage in trophallaxis (i.e. exchange of liquid food), these structures are required for the autonomous feeding from pieces of prey provided by workers (Fig. 1B,C).

Posterior to the head, the 13 segments typical of hymenopterous larvae are distinct in *D. ceylonense* (Fig. 1A). Every trunk segment is covered with 10 prominent, pointed tubercles that are located dorsally (4), laterally (4) and ventrally (2) (Fig. 1C). In Hymenoptera, the first three trunk segments (T1, T2 and T3) fuse with the first abdominal segment (A1) to become the adult thorax (Snelling 1981; Bitsch and Peeters 1991). The remaining abdominal segments form the petiole and the gaster. Interestingly, the first four trunk segments in *Diacamma* larvae (T1 to A1) are thinner (Fig. 1A) and form a functional neck, which is either bent ventrally on the abdomen or actively searching for food.

### *Morphological variations*

We have been able to observe and film larval moulting in *D. ceylonense*. The cuticle broke near the head, then moved posteriorly by muscular contractions of the larva's body. When the sloughed cuticle was attached by the posterior extremity only, a worker grasped and ate it. Since moults occur extremely quickly, it was difficult to directly determine both number and duration of instars. We therefore sought moults correlated variations among the common characters described above. The clearest changes between larvae occur



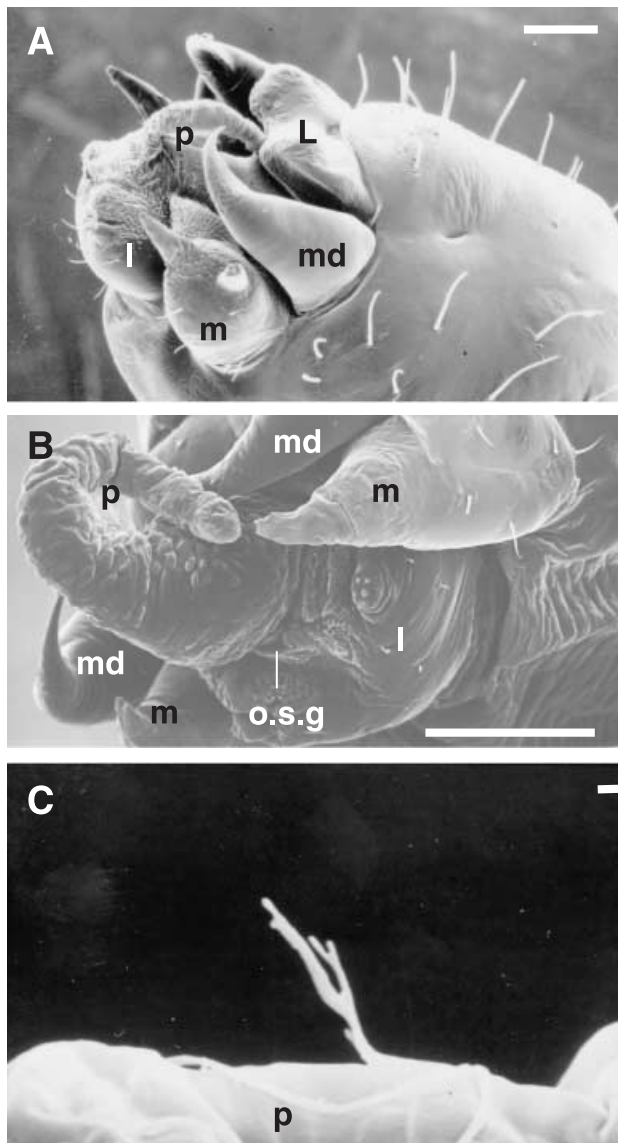
**Fig. 1**—SEM of male larvae of *Diacamma ceylonense*. —**A**. Ventral surface of a third instar larva. Because of its hook shape, it is possible to distinguish the segments A8, A9 and A10 in the centre of which the posteroventral anus is located. —**B**. and —**C**. SEM of head of larvae belonging to second and fourth instars, respectively (for third instar, see Fig. 2A). Positions of tubercles are indicated in C on the left part of the T2 segment: D, dorsal; L, lateral; V, ventral; L, labrum; md, mandibles; m, maxilla; l, labium; a, antenna; p, proboscis. Scale bars = 100 µm.

on the cuticle. First, we identified four different kinds of tubercles: (i) hemispheric tubercles with two or three proximal hair-like structures (Fig. 3A), (ii) subconic tubercles with a very short spine at the tip and two or three hair-like structures (Fig. 3B), (iii) cones with minute papillae at their extremity and two or three hair-like structures (Fig. 3C), and (iv) cones with an apical spine and three or four basal hair-like structures (Fig. 3D). Second, we observed that the dorsal surface of all segments exhibits numerous spinules. Their distribution can also be divided into four different patterns: (i) minute and round solitary spinules regularly distributed (Fig. 3E), (ii) scarce spinules roughly aligned along wrinkles (Fig. 3F), (iii) clusters of five to eight spinules forming rows (Fig. 3G) and, (iv) cuticle covered with comb-like and sharp spinules (Fig. 3H). In addition, we observed that the labium of the largest larvae (bearing conic tubercles

with spine) lacked a proboscis (Fig. 1C), unlike smaller larvae. None of these characters discriminated male from female larvae.

#### *Number and duration of instars*

On the basis of these variations, we conclude that there are four larval instars in *D. ceylonense*. Indeed, each observed larva bore only one kind of tubercle and none of them presented an intermediary shape differing from the four kinds described above, suggesting a lack of continuum in tubercle shapes. The occurrence and location of tubercles did not vary between larvae bearing the same kind of tubercles. In addition, each distinct spinule pattern is strictly correlated with each type of tubercle (Fig. 3, Table 1). Therefore we assume that each kind of tubercle and spinule pattern



**Fig. 2** —A. SEM of the mouthparts of a third instar larva. —B. Detail of the distal extremity of proboscis. —C. Detail of one multibranching hair on proboscis. L, labrum; md, mandibles; m, maxilla; l, labium; p, proboscis; o.s.g, opening of the silk gland. Scale bars = 100  $\mu\text{m}$  in A and B; 1  $\mu\text{m}$  in C.

does not change until the next moult and correspond to a single instar. The complete description of each instar is given in Table 1 and can be used as a determination key.

Finally, one clear sex-linked variation was detected: the length of pupal life, which was  $21 \pm 1$  days for females ( $n = 22$ ), as against  $30 \pm 0.5$  days for males ( $n = 11$ ). Given that the duration of embryogenesis was about 4 weeks (30 days, data unshown) and of larval development was 3 weeks (Table 1), we estimate that the total development time of

*D. ceylonense* under our laboratory conditions is around 10 weeks for females and 11–12 weeks for males.

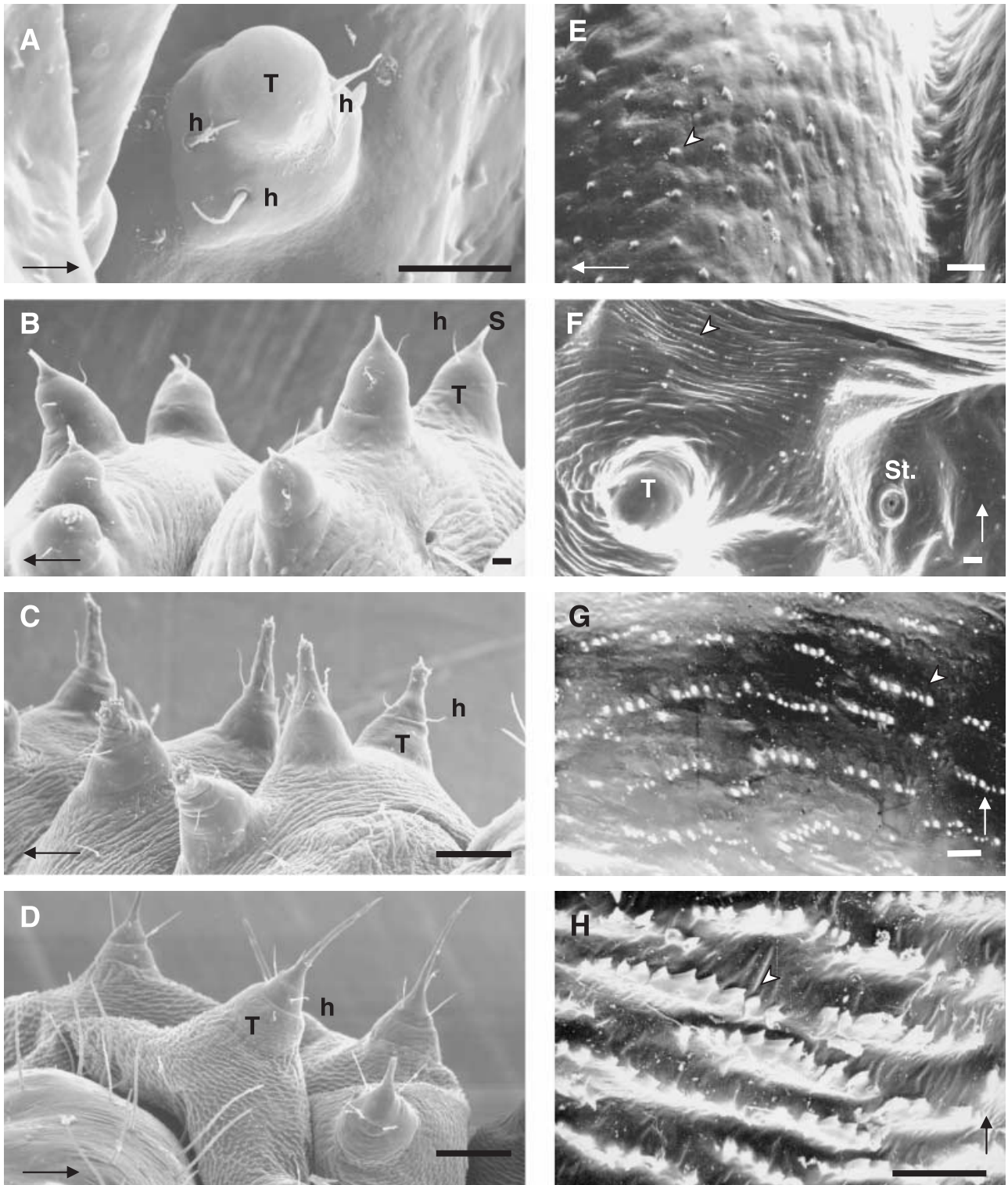
#### Development of gemmae

Larvae of *Diacamma* do not exhibit any trace of vestigial structures (leg, gonopod, wing or gemma rudiments) and imaginal discs are not exteriorly visible until their evagination at the pupal stage. In pupae, the gemma is a conical structure, unfolded and pointing outside perpendicularly to the mesonotum (Fig. 4A). Unlike the adult gemma, which is connected to the thorax by a narrow and small stalk (Fig. 4B), the pupal gemma exhibits a proximal connection to the body wall larger than the distal tip. This suggests that both gemma and stalk differentiate only at the end of the pupal stage. In addition, precise SEM observations of the dorsal surface of pupal gemmae did not reveal any trace of secretory pores.

#### Discussion




Few *Diacamma* larvae have been described: five ‘mature’ larvae of *D. australe*, four ‘mature’ larvae of *D. scalpratum*, and one ‘young’ and four ‘mature’ larvae of *D. rugosum* sp. from the Philippines (Wheeler and Wheeler 1952). Based on their tubercle shape, the mature larvae of *D. ceylonense* are closely similar to those of *D. rugosum*, consistent with the fact that both species belong to the *rugosum* group, defined on adult characters by W.L. Brown (unpublished monograph). Here we describe four distinct instars in *D. ceylonense*. It appears that four is a common number of larval instars in ants, but interspecific variation has been observed, from three instars (*Creumatogaster stadelmanni*, Delage-Darchen 1972) to six instars (queen of *Camponotus aethiops*) (see Masuko 1990 for review). In various Apidae and in *Vespa* wasps, the number of moults is difficult to determine because the larvae grow in cells, but five instars are generally observed (Michener 1974).

In some studies, instar determination is based on quantitative measures, such as head width, body length, or body shape (Gotoh *et al.* in press; Sameshima *et al.* 2004). However, temperature, food supply and season may induce great variations in larval weight within a given species (Wheeler and Wheeler 1979; see Discussion in Delage-Darchen 1972; Petralia and Vinson 1979). Although our data show significant differences of body weight between instars, we occasionally found larvae belonging to one instar (based on morphology) to be heavier than some larvae of the following instar (see ranges in Table 1). As a consequence, the instar of a given larva cannot be unambiguously assessed by its body weight or length alone. Because larval moults are basically a change of cuticle, variations in cuticular processes are likely to discriminate instars in all holometabolous insects (Wigglesworth 1972). In the case of *D. ceylonense*, tubercles and spinules make it possible to determine the developmental stage, but do

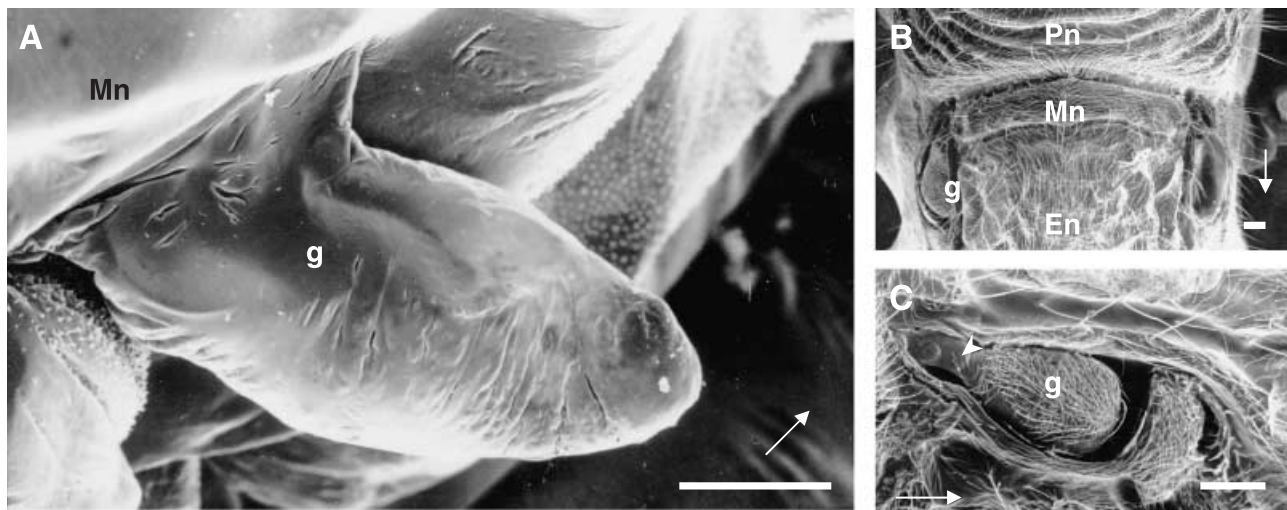


**Fig. 3**—SEM of larval cuticles (first segments after the head). —**A–D**. Tubercles of instars 1, 2, 3 and 4, respectively. —**E–H**. Cuticle surface of instars 1, 2, 3 and 4, respectively. T, tubercle; h, hair-like structures; S, spine; St, stigmata; arrowhead, one spinule. Scale bars = 100  $\mu$ m in C and D; 10  $\mu$ m in A, B, E, F, G and H. Arrows indicate orientation from anterior to posterior.

**Table 1** External morphologies of the four larval instars in *Diacamma ceylonense*, durations of instars and average weight

	Keys for instar determination				Description	
	Tubercle shape	Spinules	Body shape	Proboscis?	Duration (days)	Weight (mg) <sup>1</sup>
Instar I ( <i>n</i> = 7)	hemispheric Fig. 3A	numerous, solitary and regular Fig. 3E	 egg-like smooth	Yes	5–6	Range: 0.1–0.7 Mean ± SD: 0.37 ± 0.27
Instar II ( <i>n</i> = 7)	subconic Fig. 3B	scarce along wrinkles Fig. 3F	 clearly segmented	Yes Fig. 1B	3	Range: 0.5–3.2 Mean ± SD: 1.94 ± 0.87
Instar III ( <i>n</i> = 12)	conic with papillae Fig. 3C	numerous, clustered in rows Fig. 3G	 'pachycondyliform' (Wheeler and Wheeler 1964)	Yes Fig. 2A,B	5	Range: 3.0–12.4 Mean ± SD: 8 ± 2.5
Instar IV ( <i>n</i> = 18)	conic with a spine Fig. 3D	comb-like Fig. 3H		No Fig. 1C	7	Range: 9–29.2 Mean ± SD: 20 ± 6.1

<sup>1</sup> Body weights are statistically different between instars (ANOVA test, *n* = 4 groups, *F* = 53.89, *P* < 0.0001).

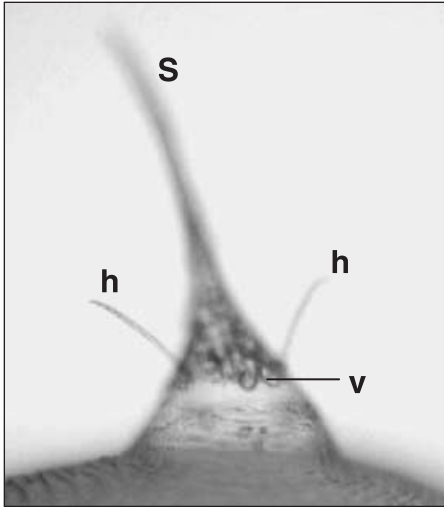


**Fig. 4**—**A.** SEM of the dorsal surface of a pupal gemma. —**B.** Dorsal view of a worker mesonotum whose right gemma has been experimentally removed from its pleural cavity (gemmarium) and whose left gemma is intact. —**C.** Right gemma folded in its gemmarium and attached to the pleuron by a stalk (arrowhead). Pn, pronotum; Mn, mesonotum; En, epinotum; g, gemma. Scale bars = 100 µm. Arrows indicate orientation from anterior to posterior.

not provide any information about the sex of the larva. The striking disappearance of the proboscis in the final instar may relate to changes in feeding habits, although this remains to be determined.

In ants, tubercles are most common in Ponerinae but they are also found in larvae of some social allodapinine bees and polybiine wasps (Wheeler and Wheeler 1979). According to Wheeler and Wheeler (1964), mature larvae of most Ponerinae

species have tubercles of only one kind. Our observations in *D. ceylonense* lead us to assume that this is also the case for each larval stage. Wheeler (1910) suggested four functions for tubercles: support (to keep the body surface away from the substrate), defence (against cannibalism between larvae), attachment to ceilings and secretion of substances attractive to workers. Our laboratory-based observations favour the last hypothesis. *Diacamma* larvae are hardly ever observed on



**Fig. 5**—Fourth instar tubercle seen under an optical microscope ( $\times 200$ ) with a specific focus which makes several vesicles (V) visible by transparency. h, hair-like structure; S, spine.

the chamber floor and are frequently held in the mandibles of the workers. Vesicles are distinguishable through the thin and transparent cuticle of tubercles, just at the base of their spine (Fig. 5) and could play a role in chemical recognition by workers. Further studies are needed to evaluate whether spinule patterns regularly exhibit sufficient variation to provide a general criterion for instar discrimination in ants, particularly in species lacking tubercles or body hairs.

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