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Original Article

Larval hemolymph feeding and hemolymph taps in the ant *Proceratium itoi* (Hymenoptera: Formicidae)

Keiichi Masuko

Abstract

Queens of the ant species *Proceratium itoi* (Forel, 1918) ordinarily obtain their nutrition through a phenomenon known as larval hemolymph feeding (LHF), whereby they feed on the hemolymph of final (4th-) instar larvae of their own offspring. Each larva has four pairs of specialized structures on the dorsal integument on the 2nd through 5th abdominal segments. Each structure consists of a small cuticular area with a shallowly cracked surface, resembling the surface of a corncob. Because of these cracks, the queen can break open the cuticular surface by mandibular pinching and intake the hemolymph from the resulting openings. This structure can be considered the *P. itoi* larval hemolymph tap and is the second confirmed case of such an organ in ants (the first was in *Leptanilla*). The queen in a developed colony depends exclusively on larval hemolymph for her nutrition, but in an incipient colony, with a small population, the queen suppresses LHF and feeds only on prey. Meanwhile, the workers feed on prey and seldom perform LHF except when the colony is starving. The wounds on the larval integument close shortly after LHF by hemolymph coagulation, and the larvae are repeatedly subjected to LHF, exhibiting multiple scars characteristic of LHF on their dorsa. Similar scars are also found on the dorsa of many prepupae, indicating that they can survive LHF and mature.

Key words: Ant, *Proceratium itoi*, queen, larva, hemolymph feeding, hemolymph tap, coagulation.

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Keiichi Masuko, Biological Laboratory, Senshu University, 2-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa Prefecture 214-8580, Japan. E-mail: kmasuko@isc.senshu-u.ac.jp

Introduction

Larval hemolymph feeding (LHF), or simply hemolymph feeding, is the term coined to denote the phenomenon in which adult ants, especially queens, feed regularly on the hemolymph of their own larval progeny (Masuko 1986). Only older larvae in the final instar are affected and, despite the apparent harm resulting from LHF, many of them mature and successfully eclose as adults since most prepupae have the scars of LHF on their dorsal surface (Masuko 1986). This phenomenon may be thought of either as nondestructive cannibalism by adults on larvae or a form of nutrient transfer from larvae to adults in a social insect colony. Two forms of hemolymph feeding can be distinguished: a crude, perhaps ancestral form and a more derived / specialized form. In the crude form, the queen uses the mandibles to wound or puncture the integument of the dorsum of a larva and imbibes the hemolymph leaking from the resulting openings. However, the injuries close rapidly, likely due to hemolymph coagulation, and the same larvae can be repeatedly employed for feeding; consequently, the larval dorsum has dark scars characteristic in appearance and location. The other form of LHF involves a specialized hemolymph-discharging organ on the larval body surface. Histological analysis in a previous study revealed that this organ is a duct-like structure connecting the outer surface and the internal body cavity (hemocoel), and the author called the organ a "larval hemolymph tap" (Masuko 1989). This type of specialized LHF has only been observed in the ant genus *Leptanilla*. In contrast, the first type has been observed in several genera, mostly belonging to the poneroid subfamily Amblyoponinae (*sensu* Borowiec & al. 2017), namely, *Stigmatomma* (= *Amblyopone*) (see Masuko 1986), *Myopopone* (see Ito 2010), *Mystrium* (see Wheeler & Wheeler 1988), *Onychomyrmex* (F. Ito, unpubl.), *Prionopelta* (see Ito & Billen 1998), and *Adetomyrma* (see Saux & al. 2004). This puncturing type of hemolymph feeding has also been documented in the formicoid subfamilies (*sensu* Borowiec & al. 2017); in *Calyptomyrmex* in the subfamily Myrmicinae (see Ito 2001) and *Gnamptogenys* in the subfamily Ectatomminae (see Ito & Gobin 2008), workers and queens, respectively, were observed to perform this behavior. Finally, hemolymph feeding has been described briefly

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Fig. 1: Spheroidal chambers of a *Proceratium itoi* nest excavated in the soil. Nests of this species consist of multiple subterranean chambers like these, where the ant brood (left arrow) and prey eggs (right arrows) are placed separately.

only for three Japanese species in the poneroid subfamily Proceratiinae, that is, *Proceratium itoi* (Forel, 1918), *P. japonicum* Santschi, 1937, and *P. watasei* (Wheeler, 1906) (Masuko 1986), but no further details were reported in the study. Therefore, the aims of the present study were to detail hemolymph feeding in *Proceratium* ants, using *P. itoi*. We found that, although workers feed on prey, queens are dependent exclusively on hemolymph feeding for their nutrition once a colony has developed, and that each larva has four pairs of hemolymph taps on the dorsal integument of the abdominal segments. These specialized structures are initially intact (closed), but are broken open by the queen's mandibular gnawing. This is the second instance of hemolymph taps confirmed in ants; however, the tap structures are completely different between *Leptanilla* and *Proceratium*. In this study, we characterized LHF in *P. itoi*, and report on the larval stage subjected to LHF, behavior of the queen, and morphological structure of the hemolymph taps.

Material and methods

Ants: *Proceratium itoi* is a reddish-brown ant; the workers are approximately 3 mm and the queen 4 mm long (Onoyama & Ogata 1989, Japanese Ant Database Group 2003). In this study, colonies of *P. itoi* were collected from an evergreen broad-leaved forest in Cape Manazuru (35.144° N, 139.154° E), Kanagawa Prefecture, central Japan. The ants were brought to the laboratory, counted, and then cultured or preserved either in 80% ethanol or Kahle's solution (Barbosa 1974), depending on the intended purpose.

This species is subterranean and the nest is constructed directly in the soil, mostly at depths of 10 - 15 cm (Fig. 1). The organization of most colonies at Manazuru is monogynous, but approximately 10% of the collected colonies contained multiple queens (K. Masuko, unpubl.). Ants of

Fig. 2: Scars on the larval integument of *Proceratium itoi*. (a) Dorsal view of a fourth-instar larva (prepupa). A pair of scars are present in each posterior region of the 2nd to 5th abdominal segments (marked as II - V). Scale bar 0.5 mm. (b) Whole-mount preparation of a fourth-instar larva. The left hemolymph tap is broken open on the 4th abdominal segment. Stained with acid fuchsin.

the genus *Proceratium* are known as specialized predators of arthropod eggs (Brown 1957, 1958), and the field data from Manazuru forest revealed that *P. itoi* predates on eggs of Chilopoda, Hemiptera, and Opiliones (K. Masuko, unpubl.). This predation and other aspects of the ecology of this ant will be published elsewhere.

Ant rearing: Three monogynous colonies collected from Manazuru were cultured in the laboratory to inves-

Tab. 1: Percentage of time spent by *Proceratium itoi* queens in each behavior. [a]Percentage of time in which prey was present in the brood chamber compared with the total sample time. [b]Composition of the colonies when the behavior of the queens was observed in the laboratory.

	A	B	C	D
Queen	81-68	84-36	14-5	14-5
Colony stage	Developed	Developed	Incipient	Developed
Behavior				
Larval hemolymph feeding	2.6	3.2	0.0	4.8
Feeding on prey	0.0	0.0	3.6	0.0
Resting	53.3	44.5	58.6	51.8
Self-grooming	4.4	7.8	3.8	9.6
Being groomed	1.0	2.0	0.0	1.0
Others	38.7	42.5	34.0	32.8
Total	100.0	100.0	100.0	100.0
Sample time (h)	18.4	15.2	15.0	15.0
Prey availability (%)[a]	100.0	100.0	100.0	100.0
Colony composition[b]				
Number of queens	1	1	1	1
Number of workers	35	31	3	31
Number of larvae	c. 40	c. 50	11-12	c. 100

tigate the behavior of the queens. Two colonies (nos. 81-68 and 84-36) were collected in 1981 and 1984, respectively, and queen behavior was analyzed in 1984. An additional colony (no. 14-5) was collected in 2014 and studied in 2015. The ants were housed in styrene observation nests (105 × 113 × 20 mm or 65 × 100 × 22 mm, depending on the size of the housed population); the bottom of each nest was covered with plaster of Paris mixed with activated carbon powder. Brood chambers were excavated in the center of the plaster floor, and the tops of the terraria and brood chambers were covered with clear glass. The ants were easily reared on eggs of the spiders *Pardosa astrigera* L. Koch, 1878 and *Parasteatoda tepidariorum* (L. Koch, 1841). When the spider eggs were unavailable, the hatchlings (the first-instar larvae) of the mealworm *Tenebrio molitor* Linnaeus, 1758 were used as prey. When such small soft-bodied mealworms were supplied alive in the foraging arena, they were immediately retrieved into the brood chamber by workers and consumed by the larvae. Prepupae and pupae of other ant species like *Myrmecina nipponica* Wheeler, 1906 were also used as prey, although infrequently. The colonies were kept at 21 - 26 °C in the laboratory.

Measurement of head and body width of scarred larvae and quantification of scars: As the larger, fourth-larval instar individuals are invariably the ones subjected to LHF (see below), the body size and the degree of LHF-induced scarring, which reflect the age and frequency of LHF, were examined in the fourth-instar larvae present in the two colonies when they were collected in the field. Both colonies were mature. One (no. 84-72) was collected on 7 July 1984 and contained one queen, 34 workers, 20 eggs, and 165 larvae; the other (no. 84-114) was collected on 26 July 1984 and contained one queen, 199 workers, 47 eggs, 290 larvae, 255 prepupae, and 12 pupae (11 gynes and one worker). All these specimens were preserved in Kahle's solution (later transferred to 80% ethanol) immediately after being counted in the laboratory. From each colony, 60 - 70 fourth-instar larvae (not including prepupae) were examined for head width at 160× and the width of the 3rd abdominal segment at 63× magnification, using a stereomicroscope (Olympus Model X-II) equipped with an ocular micrometer with a precision of 12.8 µm and 32.3 µm, respectively (based on calibration). As most of these larvae had multiple scars characteristic of LHF on their dorsa, and the scars varied in size or area (Fig. 2a), it was necessary to quantitate the total extent of scarring for each larva. For this, degrees of scarring were classified into three arbitrary classes: light, medium, and heavy, scored as + 1, + 2, and + 3, respectively. Thus, each larva had a total score according to the number of scars and degree of scarring. In addition to the scars included in these three classes, faint scratches could be observed on the larval body surface; these were minute, but seemingly caused by biting of the queen (see below). As they appeared to be superficial injuries that did not puncture the integument, they were not included in the scoring.

Queen behavior: From a previous study on *Stigmatomma* (= *Amblyopone*) *silvestrii* Wheeler, 1928 (Masuko 1986), it was suggested that, as in the case of *S. silvestrii*, the *Proceratium itoi* queen of a developed colony may also depend exclusively on hemolymph feeding for her nutrition; in contrast, the queen of a founding or

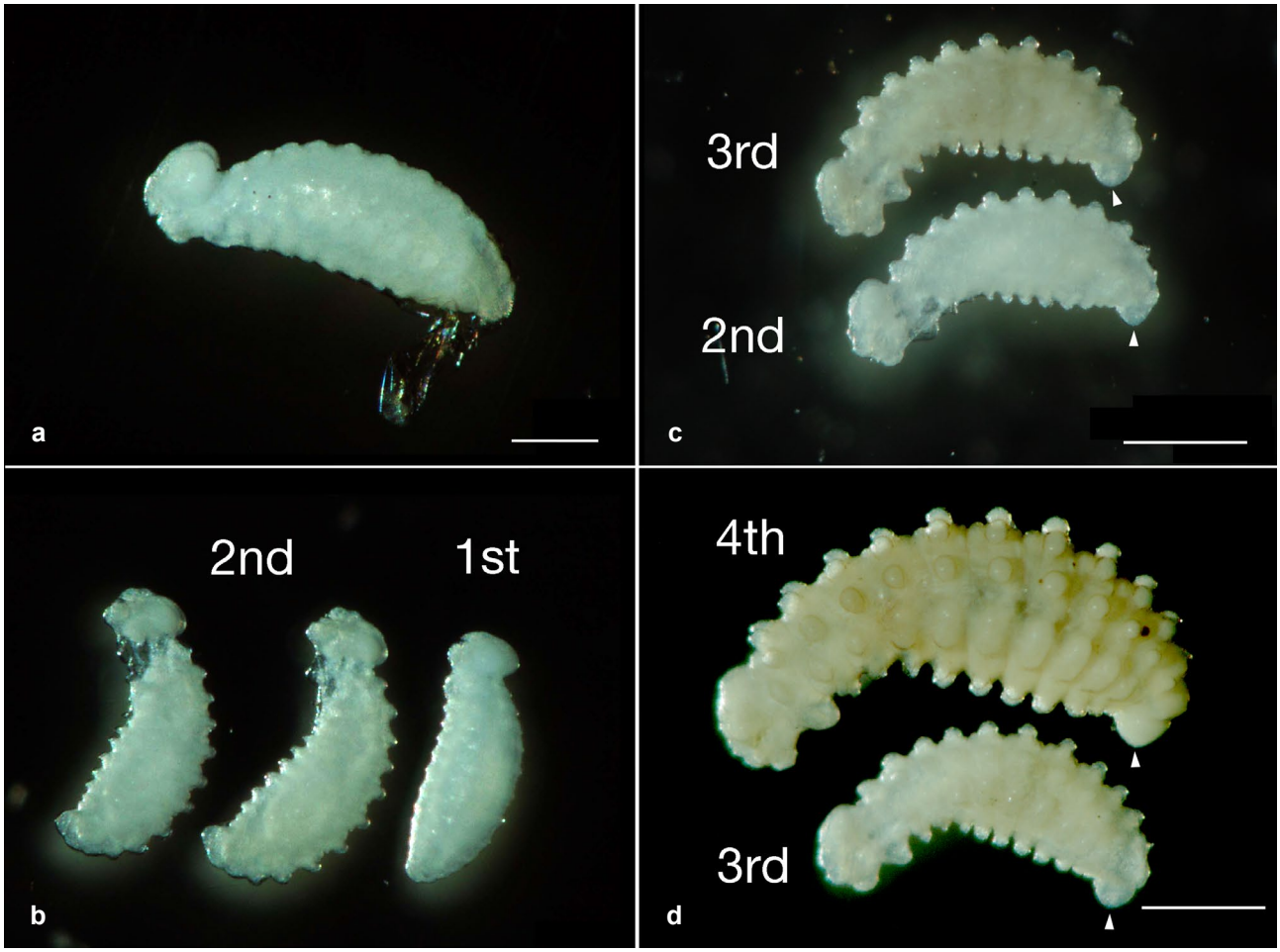

Fig. 3: Larval instars of *Proceratium itoi*. The larval stage of *P. itoi* consists of four instars. Between-instar differences in the morphology of the 10th abdominal segment (arrowheads) and the dorsal and ventral protuberances are apparent in side-by-side comparisons of the entire lateral view. (a) First larval instar. The egg membrane is attached to the posterior end of the first instar. (b) First and second larval instars. (c) Second and third larval instars. (d) Third and fourth larval instars. Scale bars: 0.25 mm in (a), and 0.5 mm in (c) and (d). No scale in (b).

incipient colony would feed on prey and suppress LHF to enhance the development of the larvae. To test this, a behavioral time budget was performed for each of the three *P. itoi* queens that occupied colonies at different stages of development. The census of these colonies at the time of the behavioral studies is given in Table 1. Observations were made under a stereomicroscope attached to a swing arm (10 - 40× magnification). The behavioral repertoire of *P. itoi* queens had been established by recording notes on audio tapes before starting the quantitative analysis. Thereafter, the observed behaviors were logged directly onto a portable microcomputer (Epson HC-20). For the queens of developed colonies (nos. 81-68 and 84-36), behavior was assayed by the "focal animal sampling" method (Altmann 1974) over a total of more than 15 h, consisting of 57 - 110-min episodes (repeated during 11 - 22 September 1984, for queen 81-68, and 16 - 30 June 1984 for queen 84-36).

When the queen of an incipient colony (no. 14-5) was studied, a different method, "point sampling" (Dunbar 1976), was used due to technical reasons related to the microcomputer. In this procedure, behavior was recorded on a worksheet at intervals of 30 s during each 1 h sampling session, making a total of 120 data points per h. These sessions were repeated 15 times from 18 March to 4 April 2015; therefore, the behavioral time budget of the queen was based on 15 h of observation. This queen was collected on 11 August 2014 with a colony at a relatively early stage of development containing the queen, one adult worker, seven eggs, eight larvae, one prepupa, and four worker pupae. A subsequent shortage of arthropod eggs to feed the colony retarded colony development in the laboratory and, when the behavioral study was started (18 March 2015), the colony contained only the queen, three adult workers, two eggs, 12 larvae, and one prepupa. Consequently, the colony was still in the incipient stage.

After the initial behavioral analysis, colony 14-5 developed successfully, containing more than 30 workers by August 2017. To confirm the change in the feeding characteristics of the queen, her behavior was again observed for 15 h with the same procedure used for the incipient stage. During the study period (14 - 22 August 2017), the colony

contained the queen, 31 adult workers, and approximately 15 eggs and 100 larvae; no pupae or prepupae were observed. Data for all these behavioral studies were collected between 09:00 and 23:00 hours.

SEM, histology, and whole-mount preparations: For morphological and anatomical studies, the larval specimens were obtained from colonies collected from 1981 to 1987; they were preserved first in Kahle's solution, then transferred to 80% ethanol. Some specimens were examined with a scanning electron microscope (SEM) (Keyence VE-8800). Before the SEM examination, the specimens were further dehydrated with a series of ethanol dilutions and treated with hexamethyldisilazane (Nation 1983, Masuko 1990). After air-drying, the specimens were mounted on stubs and coated with gold-palladium. A total of 52 larvae were observed (eight first-instar, nine second-instar, 14 third-instar, and 21 fourth-instar).

For histological examination of the hemolymph taps on the larval body, the preserved larvae were further dehydrated in a series of ethanol dilutions with propylene oxide, before embedding in Agar low viscosity resin (Agar Scientific). Sections 0.5 - 0.8 µm thick were obtained using a diamond knife, then stained in a 1% methylene blue / 1% borax solution. Whole-mount preparations were also made to examine closely the wounds in the cuticular integument (the method in Masuko 2017). Sections and whole-mounts were examined using compound microscopes (Olympus Vanox and BH2) and morphological details were imaged with a digital camera (Shimadzu Moticam-2500).

Results

LHF-subjected larvae and scar quantification: The larval stage of *Proceratium itoi* consists of four instars (Fig. 3). In some species of the genus *Proceratium*, the larvae have numerous protuberances or bosses on the dorsal, lateral, and ventral body surfaces (Wheeler & Wheeler 1952). The four instars of *P. itoi* are easily distinguished by a combination of differences in the morphology of these protuberances and the 10th abdominal segment, and total body length and size of head capsule (Fig. 3). Figure 2a depicts a mature fourth-instar larva (prepupa) with numerous pigmented scars on its dorsum, and all the scars are assumed to have been made by hemolymph feeding. Cuticular melanization is known to occur at wound sites of insect integuments (Nation 2016). In this larva, the dark scars are present on the dorsum of the 2nd to 5th abdominal segments that present an invariable pattern in *P. itoi* larvae. This scar distribution is more extensive than in *Stigmatomma silvestrii*, where the scars are distributed in the two intersegmental grooves between the 1st and 3rd abdominal segments (Masuko 1986) (but see Discussion for the previous misidentification of the larval body segments for this species). However, the scars are typically present in, or close to, the intersegmental grooves in both species. The protuberances on the *P. itoi* larvae, especially the dorsal ones, are currently not thought to be related to hemolymph feeding (see below), and the function of these structures remains unknown.

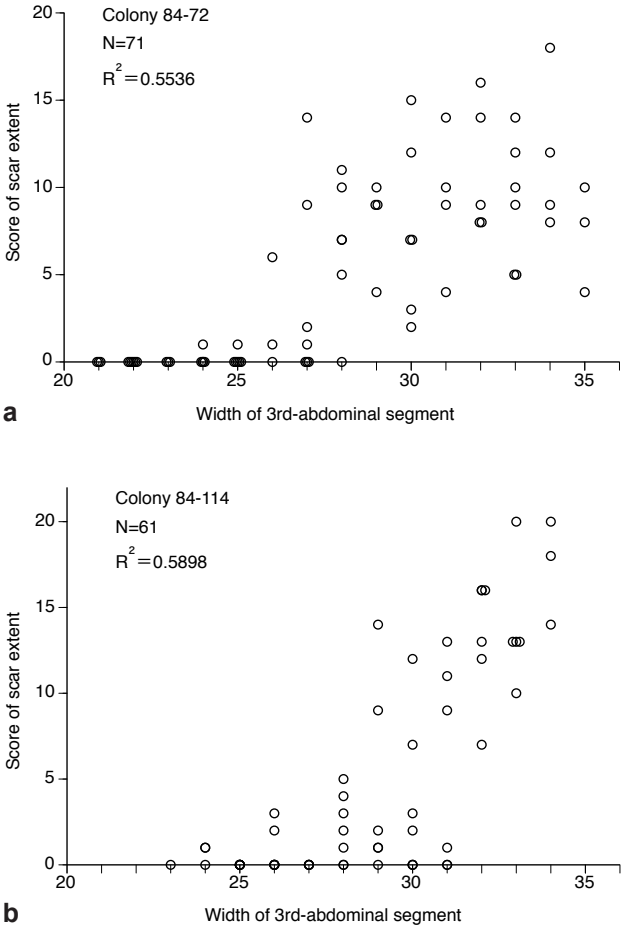

Fig. 4: Quantified extent of scarring and widths of the 3rd abdominal segment in fourth-instar larvae. Preserved specimens from two colonies (a, 84-72; b, 84-114) were studied. The *x*-axis is represented by divisions of the ocular micrometer used (1 division = 32.3 µm). The larvae present scars characteristic of LHF when they reach 0.78 mm (24 divisions) measured as the width of the 3rd abdominal segment.

Figure 4 shows the quantification of the extent of scarring on the dorsal abdominal segments of the larval specimens collected from the two field colonies. In both colonies, larvae in the early fourth instar stage, that is, smaller in size, were not subjected to LHF; instead, once they had reached a certain size, that is, 0.78 mm, measured as the width of the 3rd abdominal segment (Fig. 4a, b), they were repeatedly utilized in LHF, and the extent of scarring increased. Most or all the scars are likely to have been made by the queen as hemolymph feeding in a developed colony is almost exclusively performed by the queen (see below).

Description of LHF in *Proceratium*: When the behavior of the queen was observed in the laboratory, each bout of hemolymph feeding was always initiated with a restless walk, with active antennation, over an aggregation of larvae. In many instances, the queen did not proceed to hemolymph feeding but started self-grooming near the larval pile, followed by immobility (rest). In other instances,

Fig. 5: A *Proceratium itoi* queen performing hemolymph feeding. (a) Queen biting the lateral part of a larva while holding it with the forelegs (f) and middle legs (m); h, hind leg. (b) Queen licking the dorsal part of a larva with her lower mouthparts (arrowhead). (c) Frontal view of the mandibles of a founding queen of *P. itoi* collected from Manazuru.

however, immediately after antennating a larva, the queen put the fore tarsi on the larva and tried to seize or gnaw the larval body with her mandibles, or licked its body surface. These movements were obvious signs for the start of LHF. Despite the hemolymph taps being present only on the dorsum of the larval body, this initial handling was performed haphazardly; more specifically, when the larva was lying on its back or side, the queen initially licked and gnawed the ventral or lateral side of the larva. On this occasion, the queen did not even distinguish between the anterior and posterior ends of the larval body. In response to such tactile stimuli, it was likely that the larvae discharged a small amount of fluid from the mouth and proctodeum as, even though the droplets were hardly visible, the queen actively licked the vicinities of the mouthparts and proctodeum of the larva for a short time.

When the queen handled a larva, she did not only use the forelegs, but also extended the middle legs forward along with the forelegs and applied all of them to the lower or lateral sides of the larva (Fig. 5a); when not handling a larva, the middle legs, along with the hind legs and gastral end, were placed on the substratum to maintain posture, and the queen ceaselessly moved round the larva while licking and gnawing its surface (Fig. 5a). The mandibular gnawing was directed not only at the larval dorsum but also the ventral side; and when the larva was grasped from the side, one of the mandibles of the queen was applied to the larval dorsum and the other to the venter (Fig. 5a). Although the distal ends of the mandibles are pointed (Fig. 5c), most of the gnawing movements slid unsuccessfully over the larval surface. During this repetitive mandibular biting, the mandibular tips finally stuck and were marginally inserted into the surface of the larval dorsum. The queen immediately pinched tightly several times, and the tap was likely broken open at this time because the queen instantly stopped the mandibular movement and attached the lower mouthparts to the site (Fig. 5b). The hemolymph taps could not be observed directly with the binocular magnification used when observing this behavior, but the site where the queen attached the mouthparts corresponded exactly to the position of the hemolymph taps. Observed from the side, the lapping movement of the lower mouthparts was evident and it was likely that the queen ingested hemolymph oozing from the puncture. Each bout of hemolymph ingestion lasted for more than 5 min (mean ± SD = 464 ± 109 s, n = 7, range 305 - 610; observations on queen 14-5). After hemolymph ingestion, the queen moved a short distance from the larva and either started grooming herself or was groomed by a nearby worker.

Under well-fed laboratory conditions, most scarred larvae were observed to continue prey feeding and successfully pupated, in agreement with the observation that many prepupae collected from field colonies exhibited scarring characteristic of LHF.

LHF by queens of developed colonies: A quantitative behavioral study of the *Proceratium itoi* queens (81-68 and 84-36) in the laboratory revealed that nutrition of the queens in developed colonies is exclusively dependent on larval hemolymph (Tab. 1A, B). The colonies of both queens contained approximately 30 workers and 40 - 50 larvae when their behavior was studied. The total observation time of more than 15 h for each queen consisted of

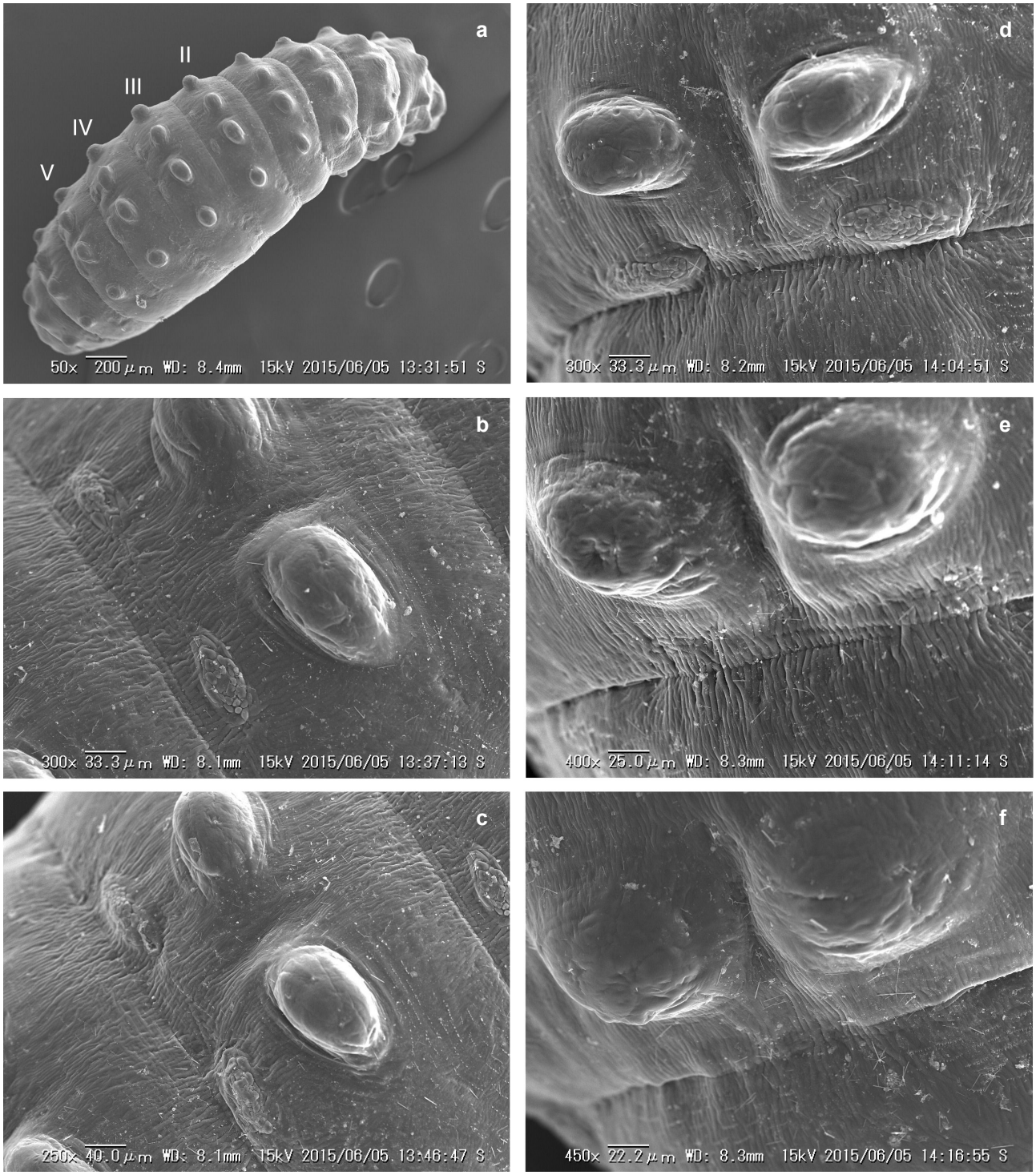

Fig. 6: Dorsal protuberances and hemolymph taps on a fourth-instar larva. Hemolymph taps are located between the dorsal protuberances and the intersegmental grooves with the following somite. No hemolymph taps exist on the 6th and 7th abdominal segments. (a) Dorsal view of a complete larva. The 2nd to 5th abdominal segments are marked as II - V. (b) Second abdominal segment. (c) Third abdominal segment. (d) Fifth abdominal segment. (e) Sixth abdominal segment. (f) Seventh abdominal segment.

60 - 110-min episodes, repeated within two weeks. During each observation episode, spider eggs were always present in the brood chamber, giving the queens the opportunity to feed freely on the prey. However, both queens continuously ignored the spider eggs and performed only LHF. No other food exchange activity, for example, feeding on worker-laid eggs or trophallaxis with workers, was observed during the study period (both the queen and workers of *P. itoi* have three ovarioles per ovary, K. Masuko, unpubl.).

Another queen, 14-5, also showed total dependence on larval hemolymph for nutrition in a colony with approximately 30 workers (Tab. 1D). During this observation,

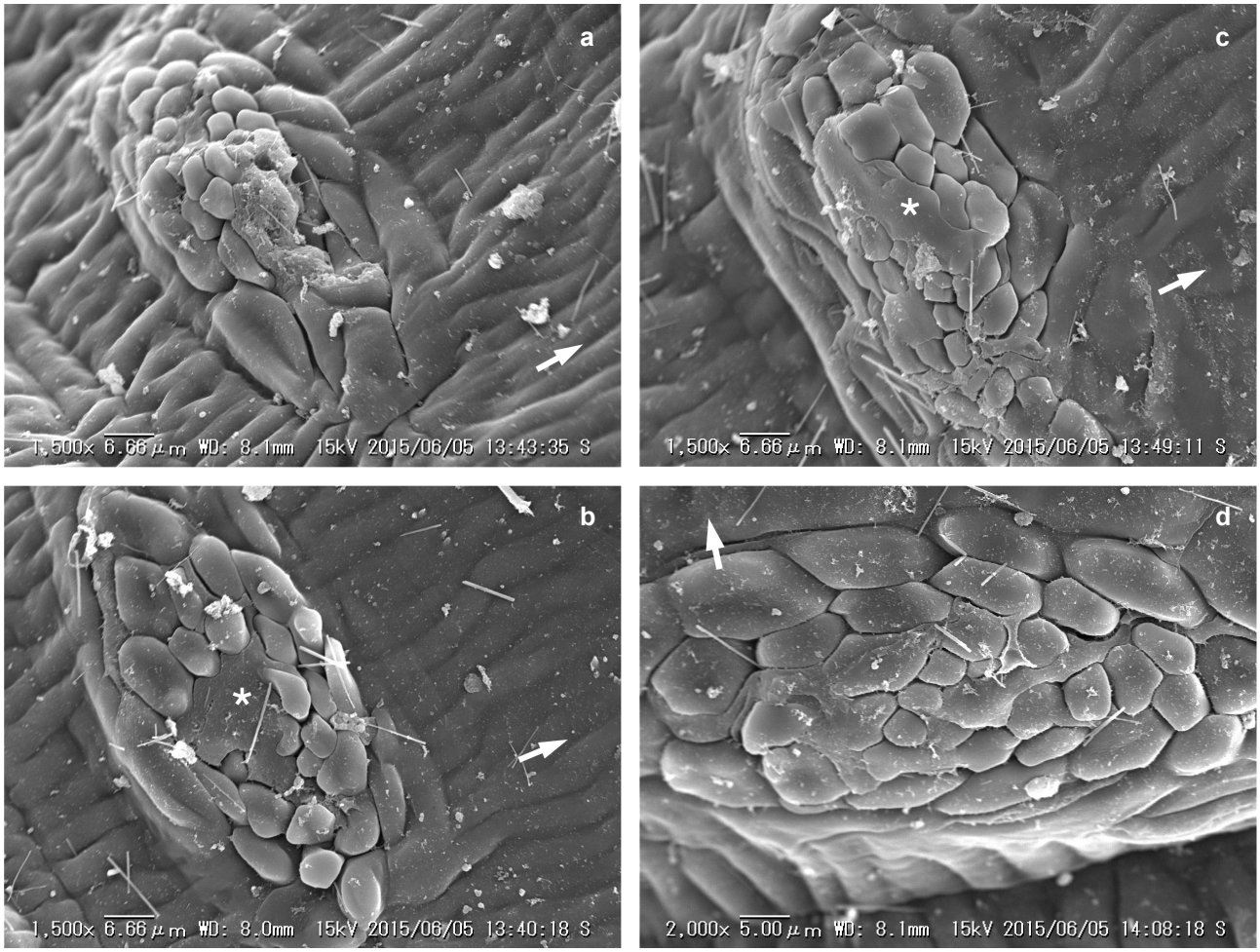

Fig. 7: Damaged hemolymph taps of a fourth-instar larva. The gaps between the cobble-like structures are filled with an unknown, uniform substance (asterisks). Arrows point to the anterior. (a) Left hemolymph tap on the 2nd abdominal segment. (b) Right hemolymph tap on the 2nd abdominal segment. (c) Right hemolymph tap on the 3rd abdominal segment. (d) Right hemolymph tap on the 5th abdominal segment.

prey (first-instar mealworms) was also always accessible to the queen. Larvae in the vicinity fed on the prey that were initially bitten and injured by the workers and then placed on the head of the larvae.

Observation of these three queens indicates that hemolymph feeding is the exclusive mode of feeding for the queens of developed *Proceratium itoi* colonies. During the observations, workers always fed on prey, and were never observed to perform hemolymph feeding.

LHF by the queen of an incipient colony: In contrast, queen 14-5 from the incipient colony that contained only three workers and approximately 10 larvae did not perform LHF, only prey feeding (Tab. 1C). At the start of the observation, the brood consisted of a prepupa, two eggs, and 12 fourth-instar larvae (the number was reduced to 11 during the study period because one larva pupated). Thus, the feeding characteristics of the queens differ greatly between early-stage colonies with small populations and developed colonies with larger populations.

To know when, and at what colony size, hemolymph feeding by the queen starts, the body surfaces of prepupae were examined occasionally under a stereomicroscope after the behavioral study on the incipient colony. No LHF scarring was found on the dorsa of the three prepupae present in the colony on 2 June 2015, when the colony contained eight workers. Similarly, no scars were observed on another two prepupae on 15 June 2015, when the colony again contained only eight workers; however, on 8 August 2015, when the colony contained 19 workers and one prepupa, the prepupa presented LHF scars on its dorsum.

Structure of the hemolymph taps: The presence of a pre-formed tap structure on the *Proceratium itoi* larval body has been overlooked, even though it has long been confirmed that *P. itoi* queens perform LHF (Masuko 1986). Instead, *Proceratium* queens were thought to imbibe larval hemolymph from new punctures made in the larval dorsum by the queen herself, as observed for *Stigmatomma silvestrii* (Masuko 1986). The possibility that hemolymph taps may be present in *Proceratium* larvae was indicated when whole-mount preparations were analyzed.

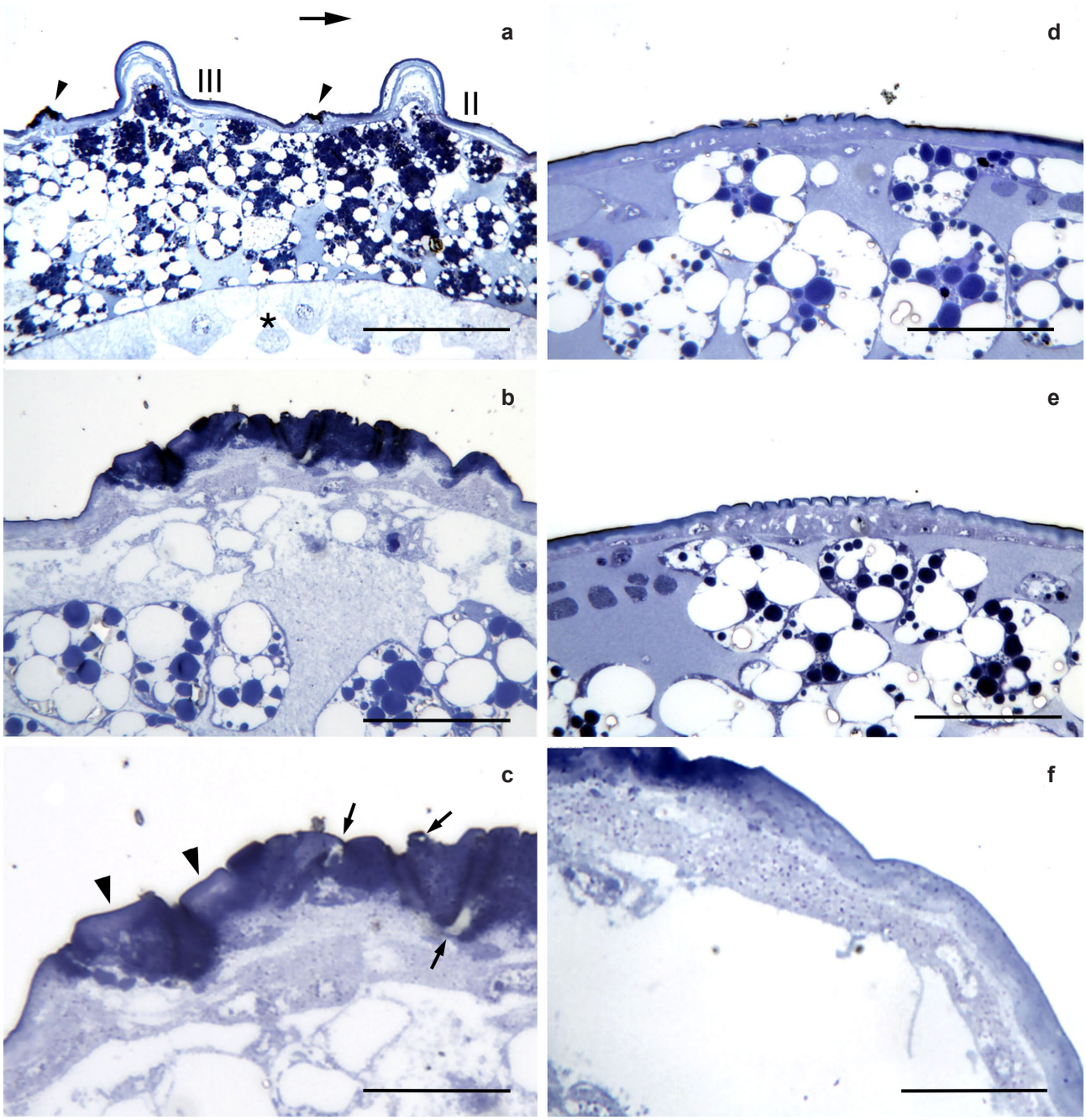

Fig. 8: Semithin sections of hemolymph taps on fourth-instar larvae. (a) Longitudinal section of the left dorsum of the 2nd and 3rd abdominal segments (marked as II and III). Arrow points to the anterior. The left hemolymph taps of the two segments are indicated by arrowheads. Asterisk marks the midgut. (b) Cross-section of the left hemolymph tap on the 3rd abdominal segment. A damaged hemolymph tap from a strongly scarred larval specimen. (c) A damaged integument. Enlargement of (b). Arrowheads indicate intact regions of the integument; strongly stained substances fill the spaces between them. Arrows point to crevices in the cuticular layer. (d, e) Cross-sections of right hemolymph taps on the 2nd and 3rd abdominal segments. Intact hemolymph taps from an unscarred larval specimen. (f) Cross-section of the periphery of the damaged integument (upper left). Same series as in (b). Small dark particles are distributed in both the cuticular layers and epidermis. Scale bars: 200 µm in (a), 50 µm in (b), 25 µm in (c), 33 µm in (d) and (e), and 20 µm in (f).

Whole-mount preparations: When whole-mount preparations were made with *Stigmatomma silvestrii* larvae, vacant openings appeared in the cuticular integument at the locations containing LHF scars (K. Masuko, unpubl.). Despite anticipating this, and even after prolonged immersion in KOH solution, numerous granule-like structures remained inside the openings in the whole-mounts of *Proceratium itoi* larvae; the structures were connected to the inner rim of the openings (Fig. 2b), and evidently formed part of the cuticular integument. Consequently,

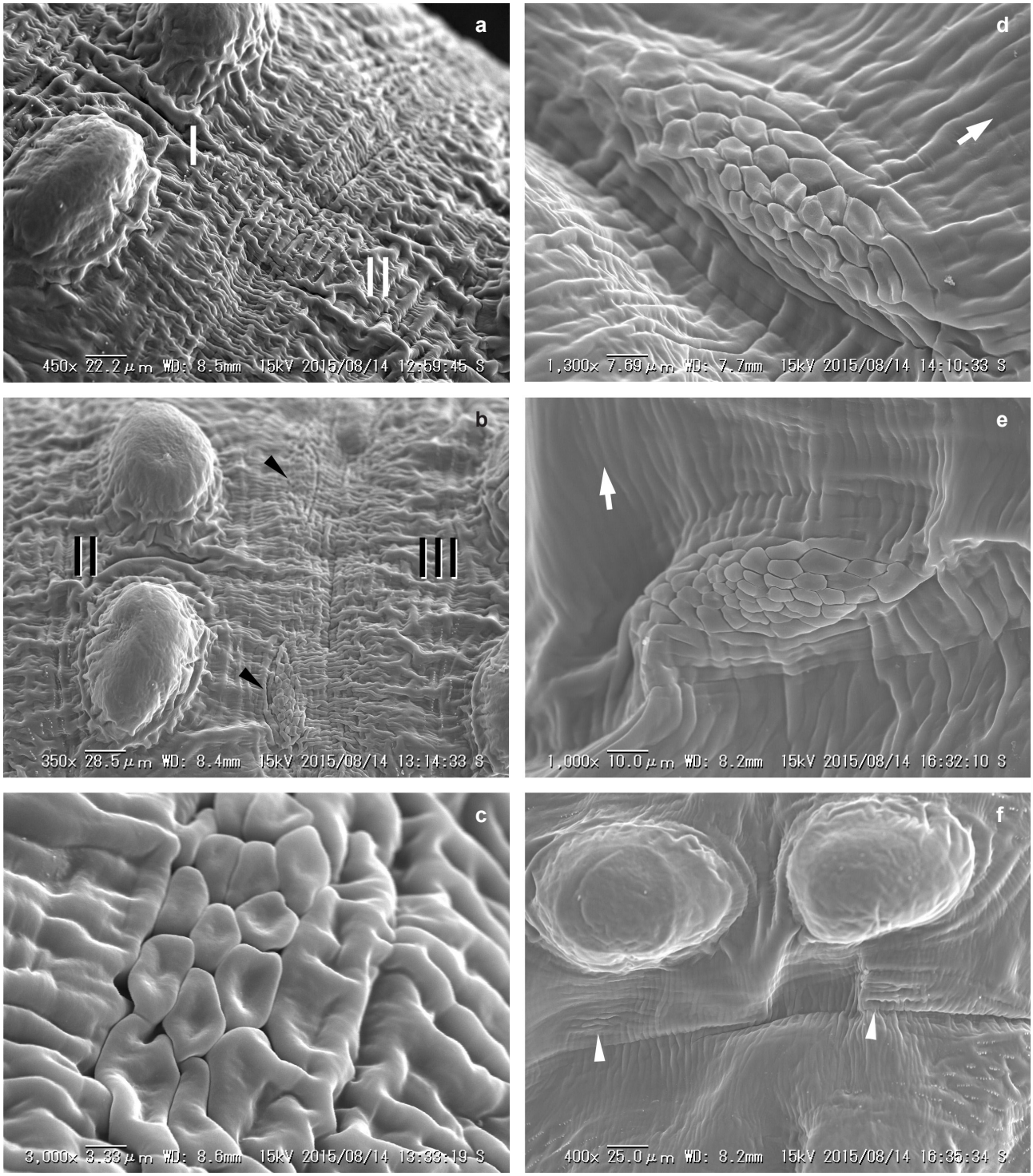

Fig. 9: Dorsal surface of the abdominal segments of a fourth-instar larva. All micrographs are from the same individual. Arrows point to the anterior. (a) First and second abdominal segments (marked as I and II). No noticeable structures exist in the posterior region of the dorsal protuberances on the 1st abdominal segment. The body surface of the larva is not fully extended soon after ecdysis. (b) Second and third abdominal segments (marked as II and III). A pair of hemolymph taps (arrowheads) are present near the intersegmental groove. (c) Surface of the left hemolymph tap on the 2nd abdominal segment. (d) Left hemolymph tap of the 4th abdominal segment. (e) Left hemolymph tap of the 5th abdominal segment. (f) Dorsal protuberances of the 6th abdominal segment. No hemolymph taps like those on the preceding somites are observed, except for faint modifications of the body surface (arrowheads).

the surface structure and internal anatomy of LHF scars were investigated closely with a SEM and with histological sections, respectively.

SEM and histology: A dorsolateral view of a complete fourth-instar larva is shown in Figure 6a. The entire body surface is covered with circular rounded protuber-

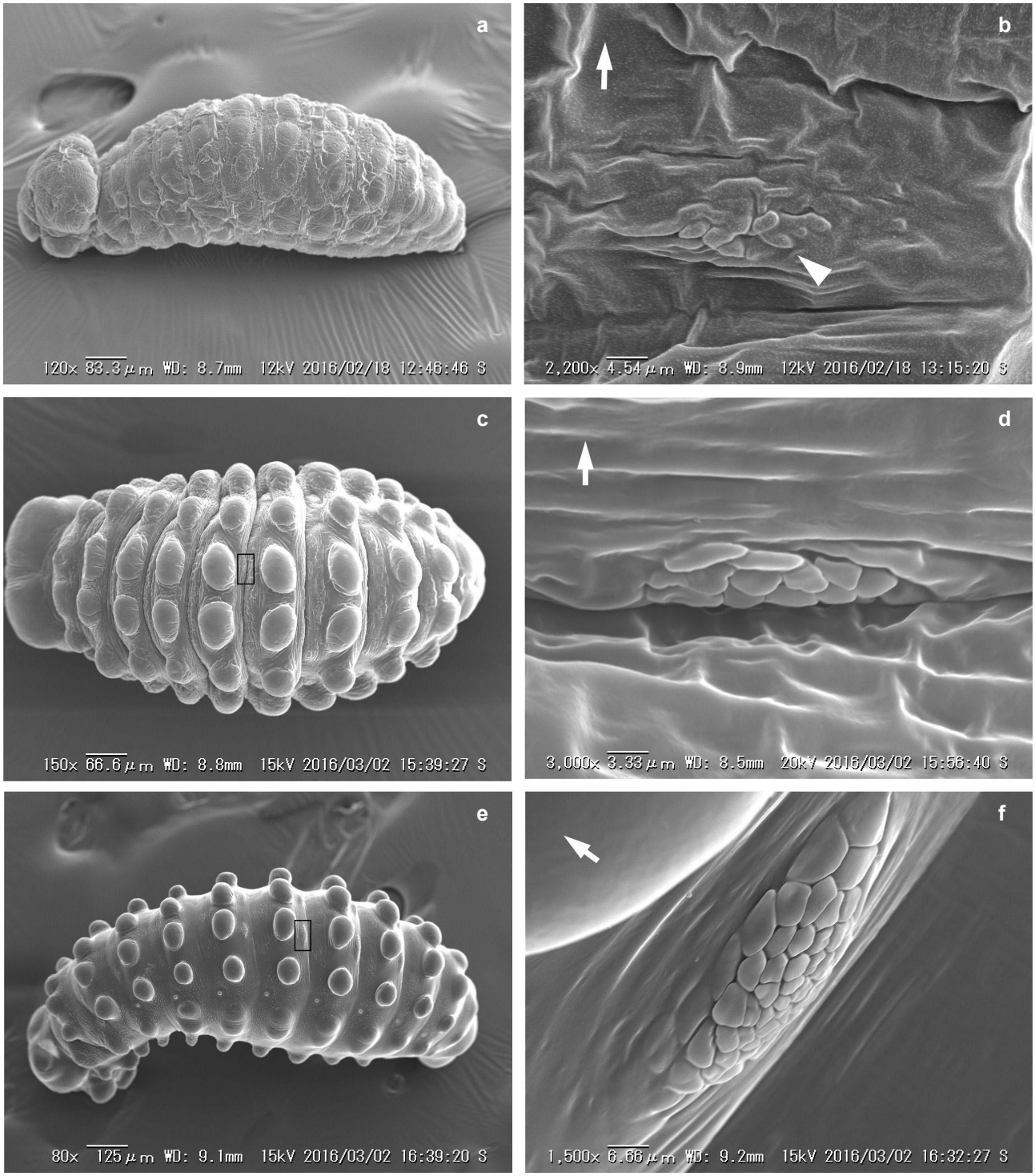

Fig. 10: First- to third-instar larvae of *Proceratium itoi*. Arrows point to the anterior. (a) Laterodorsal view of a first-instar larva. (b) Left hemolymph tap (arrowhead) on the 3rd abdominal segment of a first-instar larva. Same specimen as in (a). (c) Dorsal view of a second-instar larva. Boxed is the location of the right hemolymph tap on the 2nd abdominal segment. (d) Right hemolymph tap on the 2nd abdominal segment. Same specimen as in (c). (e) Laterodorsal view of a third-instar larva. Boxed is the location of the left hemolymph tap on the 3rd abdominal segment. (f) Left hemolymph tap on the 3rd abdominal segment. Same specimen as in (e).

ances or bosses. Conspicuous scars are located on the dorsal surface from the 2nd through 5th abdominal segments (Figs. 2a, 6a - d). They are not present on the three thoracic segments or on the 1st, 6th, and subsequent abdominal segments (Fig. 6a, e, f). The scars on each segment are paired and regularly situated behind a pair of the most dorsal bosses, and slightly anterior to the intersegmental groove. Each scar extends laterally and is raised above the body surface like a pomegranate (Fig. 6b - d). Enlarged scars are shown in Figure 7. The surface structure has an irregular

tessellated or cobblestone appearance; the "cobbles" vary in shape and size, and are identical to the granule-like structures observed in the whole-mount preparations (Fig. 2b). Notably, the gaps between the cobbles are filled with an unknown, uniform substance (Fig. 7b, c, asterisks). To further characterize the scars, histological sections were prepared and analyzed (Fig. 8).

Despite the regular spatial relationships between the dorsal protuberances and the scars (Fig. 8a, arrowheads), examination of the histological sections did not reveal any specific structural or functional connection between them. In cross-sections, the entire surface of a heavy scar was raised and lumpy, and the integument was strongly deformed and thickened (Fig. 8b). Gaps or cracks could be seen in this thickened cuticular layer (Fig. 8c, arrows), and dark-stained materials filled the gaps between the original cobble-like structures (Fig. 8c, arrowheads).

To identify structures existing before being damaged (no special structures were predicted to be present on the body surface), fourth-instar larvae were observed with a SEM shortly after ecdysis from the previous instar, thus presenting no dorsal scarring (Fig. 9). Unexpectedly, some surface structures, somewhat like the surface of a corncob, were apparent and were located exactly where scars typically appear on each of the 2nd to 5th abdominal segments (Fig. 9b - e); no such structure was apparent on the three thoracic segments or the 1st abdominal segment (Fig. 9a). Incomplete or vestigial structures could be observed on the 6th abdominal segment of some individual larvae (Fig. 9f, arrowheads). Histological observation of the structures (Fig. 8d, e) indicated that the surface of the integument neither rose nor thickened like the damaged ones; instead, the cuticular layer was regularly uneven, suggesting that the regularly interspersed crevices or depressions could be broken open by the mandibular gnawing of adult ants. Notably, the epidermal layer in that region is initially thicker than in the surrounding regions (Fig. 8d, e). The results of the behavioral observations, as well as the SEM and histological studies, indicate that these four pairs of structures on the dorsa of the 2nd to 5th abdominal segments are likely to be the organs through which adult ants imbibe hemolymph from the larval hemocoel. Consequently, these organs can be described as larval hemolymph taps like those previously described for *Leptanilla* (see Masuko 1989). Nevertheless, the structures are somewhat different between these two genera (see below).

The behavioral observations and examination of the scars on the body surface of all larval instars confirmed that only the fourth instars are subjected to LHF. To determine whether hemolymph taps pre-exist in younger instars, the dorsal body surfaces of first, second, and third instars were analyzed with a SEM (Fig. 10). Intriguingly, all the younger instars possessed hemolymph taps on each of the 2nd to 5th abdominal segments. Although the taps in the first instar appeared imperfect or vestigial (Fig. 10b), those on the third instar appeared to be almost as complete as those of the fourth instar (Fig. 10f).

Examination of semithin sections revealed that numerous small dark particles were present both in the epidermis and in the cuticular layers (Fig. 8f), and they appeared to be more concentrated in the damaged regions or periphery of the integument; in contrast, few or none were observed in non-scarred regions.

Discussion

Larval hemolymph feeding was first reported for *Stigmatomma silvestrii* under the taxonomic name *Amblyopone silvestrii* (see Masuko 1986). In a recent phylogenetic analysis based on morphology (Yoshimura & Fisher 2012), approximately 70 species (including *S. silvestrii*) were transferred from *Amblyopone* to *Stigmatomma* as new or revived combinations, while the other species remained in *Amblyopone*. Additionally, a recent molecular phylogenetic analysis (Ward & Fisher 2016) showed that *Amblyopone* and *Stigmatomma* belong to two distinct clades. LHF is likely to be also present in true *Amblyopone* as the larvae of *A. australis* Erichson, 1842 have LHF-characteristic scars on their dorsal integument (K. Masuko, unpubl.). To date, LHF has been observed in twelve ant genera from five subfamilies: *Adetomyrma*, *Amblyopone*, *Calyptomyrmex*, *Gnamptogenys*, *Leptanilla*, *Myopopone*, *Mystrium*, *Onychomyrmex*, *Prionopelta*, *Proceratium*, *Stigmatomma*, and *Typhlomyrmex* (see below). Among them, hemolymph taps were found only in *Leptanilla* and *Proceratium*. However, closer morphological and anatomical observations, as in the present study, may lead to hemolymph taps or other LHF-related organs or structures being discovered in other genera. For instance, LHF using specialized organs was reported for *Typhlomyrmex*, another genus of Ectatomminae, although the structural details remain unknown (Lacau & al. 2007).

In *Leptanilla japonica* Baroni Urbani, 1977, only the 4th abdominal segment has a hemolymph tap on each side of its posterior region (Masuko 1989). In *Proceratium itoi*, four pairs of hemolymph taps are present in the posterior region of the dorsal integument from the 2nd through 5th abdominal segments. In *Stigmatomma silvestrii*, LHF-related scarring was originally reported as being present in the two intersegmental grooves between the 2nd and 4th abdominal segments (Masuko 1986). However, a later study on the larval morphology of this species (Masuko 1990) showed that the two most anterior body regions, which had been erroneously considered as the 1st and 2nd thoracic segments, were in fact the anterior and posterior regions of the 1st thoracic segment. From this, it follows that the true 1st abdominal segment was previously mistaken for the 2nd abdominal segment and, therefore, LHF-related scars in *S. silvestrii* are now correctly considered to be located at the two intersegmental grooves between the 1st and 3rd abdominal segments. Consequently, the locations of hemolymph taps and the regions subjected to LHF are all limited to the anterior half of the larval abdomen (the abdomen of ant larvae consists of ten somites, Wheeler & Wheeler 1976). However, the reason for this bias in spatial distribution is unclear.

The structure of hemolymph taps in *Proceratium itoi* clarified in the present study differs greatly from *Leptanilla*. In the latter, a pair of naked bilateral circular areas is present on the 4th abdominal segment. In the center of this area, there is a slit-like opening that extends dorsoventrally and is attached internally to a short duct that is sharply bent and opens internally directly into the hemocoel. Hemolymph flow in the duct may initially be interrupted somewhere between the internal body cavity and the surface opening, but the queen's biting would initiate hemolymph flow in the first instance of LHF. In contrast, the larval hemolymph tap of *P. itoi* appears to consist only of a cuticular modification on the dorsal body surface. The most recent molecular phylogenetic analyses (Ward & Fisher 2016, Borowiec & al. 2017) have placed the Leptanillinae or the Leptanillinae plus Martialinae as a sister or basal group to all other ant lineages (including *Proceratium*). Therefore, the hemolymph taps in *Leptanilla* and *Proceratium* likely evolved independently.

Our behavioral study using the three *Proceratium itoi* queens in the developed colonies revealed that their nutrition was almost exclusively dependent on the larval hemolymph. However, observation of queen behavior also revealed that in each bout of LHF, the queen obtained some substance from the larvae prior to feeding on hemolymph. From the behavior of the queen, it is likely that a small quantity of fluid was discharged from the mouth and proctodeum of the larvae in response to the queen's biting. The amount of fluid transferred might be negligible as the fluid present between those larval body regions and the queen's mouthparts cannot be seen, and the queen only licked the larvae briefly. Nevertheless, the presence of such behavior may be important when considering the origin of LHF. Similarly, *Stigmatomma silvestrii* larvae were observed to discharge a transparent droplet from the mouth or proctodeum under a strong contact stimulus, or while being pinched during LHF, especially shortly after feeding (Masuko 1986). In another *Proceratium* species, that is, *P. croceum* (Roger, 1860), workers were observed to lick the larval mouth and 1st thoracic segment regularly, supposedly to obtain a minute quantity of saliva (Haskins 1930). Also in this species, the larvae were observed to be "eagerly licked for exudates" and even pinched "to hasten the flow" (Haskins 1930). Even though there was no mention of which members of the colony licked and pinched the larvae, and "the exudates" were not identified, this behavior observed in *P. croceum* is very similar to that exhibited by *P. itoi* queens prior to, or during, LHF. All these observations imply that nondestructive ways of obtaining larval hemolymph, such as LHF performed by extant ants, may have had behavioral origins; it is unlikely that a behavior resulting in a harmful breach of the integument evolved immediately when the need arose for the queen to obtain any kind of nutrient from her own larval progeny. Instead, a starving queen may have stimulated the larvae using the mandibles (pinching) or the antennae (palpating) to obtain fluids from the larval mouth and anus, as observed in extant species of *Proceratium* and *Stigmatomma*.

Indeed, several forms of nutrient exchange have evolved among members of the same colony, especially in ants of the formicoid subfamilies. One form is trophallaxis, which denotes the transfer of nutrients, mostly in liquid form, through mouth-to-mouth or anus-to-mouth feeding; the former is referred to as stomodeal trophallaxis, and the latter as proctodeal trophallaxis (Hölldobler & Wilson 1990). Stomodeal and proctodeal trophallaxis in ants have been best studied in Myrmicinae species. Ingestion of larval saliva (most likely from labial glands) by the queen was frequently observed in a myrmicine ant, *Temnothorax* (= *Leptothorax*) *curvispinosus* (Mayr, 1866) (Wilson 1974). Similarly, workers of *Myrmica rubra* (= *laevinodis*) (Linnaeus, 1758) and *Monomorium pharaonis* (Linnaeus, 1758) stimulate the larvae to discharge saliva and rectal fluid (Ohly-Wüst 1977); biochemical analyses of these fluids revealed that larval saliva contains amino acids, proteases, and carbohydrases, and the rectal fluid contains amino acids and proteins in addition to uric acids (lipases and a small amount of uric acids are also present in *Myrmica* larval saliva). Moreover, the volume of these fluids in a single discharge is not negligible (Ohly-Wüst 1977).

Finally, in ants like *Stigmatomma* and *Proceratium* that present LHF, a breach of the larval integument is not accidental but occurs regularly and repeatedly in the same larva. Histological analysis revealed numerous dark-stained particles near the cuticular region damaged by LHF. They are likely proteins that, through melanization and polymerization, may seal the injuries in combination with localized coagulation of hemolymph (Nation 2016). However, the physiology and biochemistry of LHF remains unexplored.

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