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Comparative morphology of myrmecophilous immature stages of European *Microdon* species (Diptera: Syrphidae): updated identification key and new diagnostic characters

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Abstract

Hoverflies (Diptera: Syrphidae) of the genus *Microdon* Meigen have larvae that live in ant nests where they are predatory on ant larvae. Reflecting the exceptional challenges of this very specialized lifestyle, *Microdon* eggs, larvae and puparia are highly distinctive in their morphology. Detailed descriptions of these immature stages is, however, lacking for all but a very few species, and much of this has been limited through the sole use of light microscopes. Here, using Scanning Electron Microscopy (SEM), we present detailed, comparative descriptions of the immature stages of three European *Microdon* species: *M. analis*, *M. devius* and *M. myrmicae*. Given that many adult *Microdon* species are very similar to each other in their outward appearance, we demonstrate that the morphology of their immature stages can improve our understanding of the phylogeny of the genus. We also discuss how particular adaptations of the immature morphology may allow their myrmecophilous life within ant nests. In this paper new diagnostic features are also presented to distinguish *M. myrmicae* from its sibling species *M. mutabilis*—the two are morphologically indistinguishable as adults.

Key words: Hoverflies, Social Parasites, Cryptic Species, Myrmecophiles, Ants

Introduction

Many organisms, mainly arthropods, have independently developed complex associations with ants ranging from various degrees of mutualism, commensalism, predation, parasitoidism to parasitism (Hölldobler & Wilson 1990; Thomas *et al.* 2005; Parker & Grimaldi 2014; Ivens *et al.* 2016; Lachaud *et al.* 2016). Most of these organisms, known as "myrmecophiles", are represented by several endopterygote taxa, chiefly coleopterans, lepidopterans, hymenopterans and dipterans (Hölldobler & Wilson 1990). Members of the family Syrphidae, also known as hoverflies or flower flies, are nearly ubiquitous and belong to one of the largest groups of Diptera [about 6,200 known species, 828 of which are present in Europe (Pape *et al.* 2015)]. Within the syrphid family, Microdontinae includes the highest diversity of myrmecophiles, with about 110 documented records of associations with ants (Reemer 2013). Most of these species are known to be social parasites or predators of ant broods (Rotheray & Gilbert 2011), with only one being an ant parasitoid (Pérez-Lachaud *et al.* 2014). Reflecting the presence of many peculiar apomorphies some authors have proposed to raise Microdontinae to family rank (Thompson 1972; Speight 1987). Recently thanks to the new sequencing technologies, Young *et al.* (2016) clarified several uncertain phylogenetic relationships within the syrphid family, confirming the position of the Microdontinae as the sister to other hoverfly lineages.

The most representative genus in this group is *Microdon* Meigen, the larvae of which are social parasites as-

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sociated with five ant subfamilies: Ponerinae, Dolichoderinae, Pseudomyrmecinae, Myrmicinae and Formicinae (Reemer 2013). The genus is distributed worldwide, but the subgenus *Microdon* sensu stricto is most strongly represented in the Holarctic region (Reemer & Ståhls 2013a), whereas in Europe only six species are known: *M. analis* (Macquart), *M. major* Andries, *M. devius* (Linnaeus), *M. miki* Doczkal & Schmid, *M. mutabilis* (Linnaeus) and *M. myrmicae* Schönrogge *et al.* (Doczkal & Schmid 1999; Schönrogge *et al.* 2002; Schmid 2004; Speight 2004; 2013; Gammelmo & Aarvik 2007). *Microdon* larvae are highly modified, slug–like predators of ant larvae (Garnett *et al.* 1990; Rotheray & Gilbert 2011), and represent one of the most striking examples of feeding specialization in hoverflies. As in other obligate myrmecophiles (Cammaerts 1995; Di Giulio *et al.* 2015), these larvae are able to successfully infiltrate the ant colony and feed on the ant brood, as well as gaining other benefits like shelter, favourable climatic conditions and protection from predators (Akre *et al.* 1973). The study of these myrmecophiles is challenging because they are rare, live in concealed environments (ant nests) and the interactions with their hosts are complex (Di Giulio *et al.* 2011), so they are still poorly known.

The peculiar shape of *Microdon* larvae, very different from those of other Diptera, has caused great taxonomic confusion in the past. The first to describe a *Microdon* larva was von Heyden in 1823, although he suspected it to be a slug (Reemer 2012). This false identification reflects the hemispherical shape of larva and pupa and persisted for about hundred years until Haas (1924) erased any sort of confusion (Reemer 2012).

The majority of studies on this genus are focused on the adult taxonomy, while investigations on immature stages are limited only to a few species mainly from Europe (e.g Doczkal & Schmid 1999; Rotheray & Gilbert 1999; Schönrogge *et al.* 2002; Schmid 2004; Witek *et al.* 2011; Wolton 2011; Speight & Sarthou 2017) and North America (e.g Akre *et al.* 1973; Thompson 1981; Garnett *et al.* 1990) while the developmental stages of most *Microdon* species remain undescribed. Most of the few descriptive works dealing with larvae of *Microdon* generally provide only a few morphological diagnostic details of last instar larvae and puparia (e.g. Wheeler 1908; Rotheray 1991; Gammelmo & Aarvik 2007). Scarparo *et al.* (2017) published a detailed morphological description of all immature stages of *M. mutabilis* combining light, fluorescence and scanning electron microscopy (SEM). In particular, the use of SEM techniques for the study of hoverfly immatures has proven to be particularly useful to meet modern descriptive standards and emphasize the presence of fine cuticular microstructures, often not detectable by using light microscopy (Pérez-Bañón *et al.* 2013; Andrić *et al.* 2014; Campoy *et al.* 2017; Ricarte *et al.* 2017). Especially when minute first instar larvae are analysed, SEM techniques can give important clues to infer on the evolution of larval adaptations, other than representing additional material suitable for advanced comparative studies (taxonomic, phylogenetic and morpho-functional) (Scarparo *et al.* 2017, Ricarte *et al.* 2017).

In this work, a detailed description of the immature stages of some European *Microdon* species using SEM microscopy is provided. This study is aimed at: 1) recognizing diagnostic characters to identify the species, including the cryptic ones; 2) illustrating the morpho-functional adaptations, related to the myrmecophily. As part of this research, the possibility of using some of the morphological features of immature stages to establish phylogenetic relationships within the genus is also proposed.

Materials and Methods

Examined material. This study is based on the analysis of 40 specimens of *Microdon myrmicae* (10 eggs, 10 first instar larvae, 10 third instar larvae and 10 puparia), 40 specimens of *M. analis* (10 eggs, 10 first instar larvae, 10 third instar larvae and 10 puparia) and 20 specimens of *M. devius* (10 third instar larvae and 10 puparia). Third instar larvae of *M. myrmicae* were found in three localities of south–west England, Devon, in wet grassland areas with *Myrmica scabrinodis* Nylander. Third instar larvae and puparia of *M. analis* and *M. devius* were both collected in Pisoniano (Latium, Central Italy) in April 2017, *M. devius* inside *Lasius distinguendus* (Emery) nests built underground, easily recognizable by their big earth mounds, and *M. analis* inside *Lasius emarginatus* (Olivier) nests under the bark of dead wood. In this work we also compare these three hoverfly species with specimens of *Microdon mutabilis* collected in Pisoniano (Latium, Central Italy) in 2015 and used for the description published by Scarparo *et al.* (2017). *Microdon mutabilis* images, here presented, are original and not published before. All the *Microdon* species were identified consulting the keys provided for adults and puparia by of Doczkal & Schmid (1999), van Veen (2010), Speight & Sarthou (2017), Bot & Van de Meutter (2018). In particular, for the identification of *M. myrmicae* we based our diagnosis on the host, *Myrmica* genus and the length of anterior spiracular tubercles of puparia

as described by Schönrogge *et al.* (2002). The ants were identified using the following keys: Agosti & Collingwood (1987) and Czechowski *et al.* (2002).

TABLE 1. Terminology used for the description of *Microdon* species

Terminology	
Ant	antenna
AO	anal opening
ASl	alveolate slope
Asn	anterior sensillum
Cr	crater
EF	external furrow
EL	external lobe
FS	flower–like sensilla
IJ	imbricate joint
LF	lateral furrow
LLb	lateral lobe
MA	micropyle area
MF	medial furrow
MG	medial groove
ML	medial lobe
MrB	marginal band
MrS	marginal stripe
MxPlp	maxillary palp
PC	pseudocephalon
PSn	posterior sensillum
PSprTu	posterior spiracular tubercle
ReFis	respiratory fissures
RH	respiratory hole
RP	radial projection
SC	smooth crown
SprPlt	spiracular plate
SpS	spiniform setae
SRP	simple reticulation process
VLb	ventral lobe
VP	volcano-like process
WMrB	waves of marginal band

Rearing process. Eggs and first instar larvae of *M. myrmicae* and *M. analis* were obtained in the lab from adults, as described by Scarparo *et al.* (2017). Third instar larvae and puparia found in the field were brought in laboratory. A small number were immediately preserved in ethanol 70% or 100%. The remaining specimens were kept alive and were reared in small cages provided with nest material and periodically humidified, kept at room temperature (24–27°C). Contrary to the breeding of *M. mutabilis*, described in Scarparo *et al.* (2017), this time we did not allow the parasites to have contact with the hosts, since no ant colony members were collected (except some individuals used for the identification). Adult flies, emerged from their puparia, started to mate almost immediately. We offered them pieces of wet cotton to create suitable oviposition sites. The small and white eggs were gathered with thin brushes and incubate in clean glass tubes closed with wet cotton. After the hatching no first instar larvae developed into second instars. The material is preserved in the A. Di Giulio collection (Department of Science, University Roma Tre, Rome, Italy).

Scanning Electron Microscopy (SEM). Eggs, larvae (first and third) and puparia of *M. myrmicae*, *M. analis,*

M. devius and *M. mutabilis* were fixed in 70% ethanol, dehydrated by passing through a graded ethanol series, from 70% to 100%, critical point-dried in a Balzer Union® CPD 030 unit, and gold coated in an Emitech® K550 unit for avoiding charging effects. The samples were examined with the Dual–Beam Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the L.I.M.E. (University of Roma Tre, Rome, Italy).

Acronyms. In the description we used the terminology and nomenclature of anatomical parts proposed by Courtney *et al.* (2000) for the larvae of Diptera and those of Garnett *et al.* (1990) and Scarparo *et al.* (2017) for larvae of *Microdon* (see Table 1).

Results

Shared features amongst *M. myrmicae*, *M. analis* and *M. devius*

Eggs

Elongate, ovoid, circular in transverse section, slightly tapered toward anterior apex, white in colour. Micropyle funnel-shaped, with smooth internal surface. Chorion entirely covered with distinct raised microsculpture, composed of many volcano-like processes, regularly spaced, with a deep, smooth apical depression ("crater") and alveolate steep slopes, deeply wrinkled, set on smooth stellate base, showing radial ridged projections; the incision between two adjacent basal projections semicircular; chorionic processes densely packed in jigsaw puzzle configuration, separated by deep grooves.

First instar larvae

Body features. Body whitish, suboval in dorsal view, flattened, slightly convex dorsally, with conspicuous pseudocephalon, partially or totally retractable. Anterior part slightly narrower than posterior and bearing two raised lobes. Dorsal surface rough, bumpy, transversely corrugated, deeply marked by subequal, conical, rugulose structures. Thoracic and abdominal tergites fused and not recognizable. Longitudinal grooves present dorsally dividing dorsal body surface into longitudinal fields. Dorsal and ventral surface with regularly spaced "flower-like" sensilla. Ventral surface wide, soft transversally multi-folded, markedly furrowed by a deep, longitudinal, medial groove, running along abdominal sterna and surrounded by a marginal stripe covered by elongated hairs. Anal opening wide, transverse, subtriangular. ***Pseudocephalon.*** Anterior part with two anterodorsal antennomaxillary lobes, bulging and distinctly separated one another, each apically bearing a 2–segmented antenna and 1–segmented maxillary palpus with longitudinal digitiform sensillum subapically. Ventral part of pseudocephalon with two lateral labial lobes representing the walls of a medial mouth (atrium); floor of atrium delimited by a small ventral lobe; two pairs of sensorial organs present on dorsal surface of pseudocephalon, one anterior and one posterior; sensory organs on posterior pair closer to the midline than anterior pair. Ventral surface of pseudocephalon totally covered by a dense carpet of thin trichoid structures. ***Posterior spiracular tubercle.*** Impair, distinctly sclerotised, light brown, contrasting with the whitish colour of the body, emerging perpendicularly from posterodorsal part of abdomen, with apical part wider than basal, and medially incised. Main part of tubercle furrowed longitudinally, both anteriorly and posteriorly, by a deep longitudinal groove separating two subparallel structures, each circular in section. Surface of spiracular tubercle with peculiar microsculpture, covered by imbricate, sclerotised scales; dimensions of scales decreasing basally. Apex of tubercle with two smooth plates, each with 1–2 respiratory narrow fissures that communicate with the tracheal trunks. ***Marginal band.*** Undulated fringe of elongated, parallel, radially projecting processes, surrounding the whole perimeter of the body except for the anteromedial furrow, distinctly separating dorsal from the ventral side of the body. Length of processes regularly varying, showing a variable number of waves on each side. At the apices of waves, the longest processes appearing thicker as a result of partial lateral fusion of two adjacent simple processes dorsally bearing one apical and one subapical spiniform seta. Each simple process composed of a stem, varying from elongate to short, and an apical fringed brush.

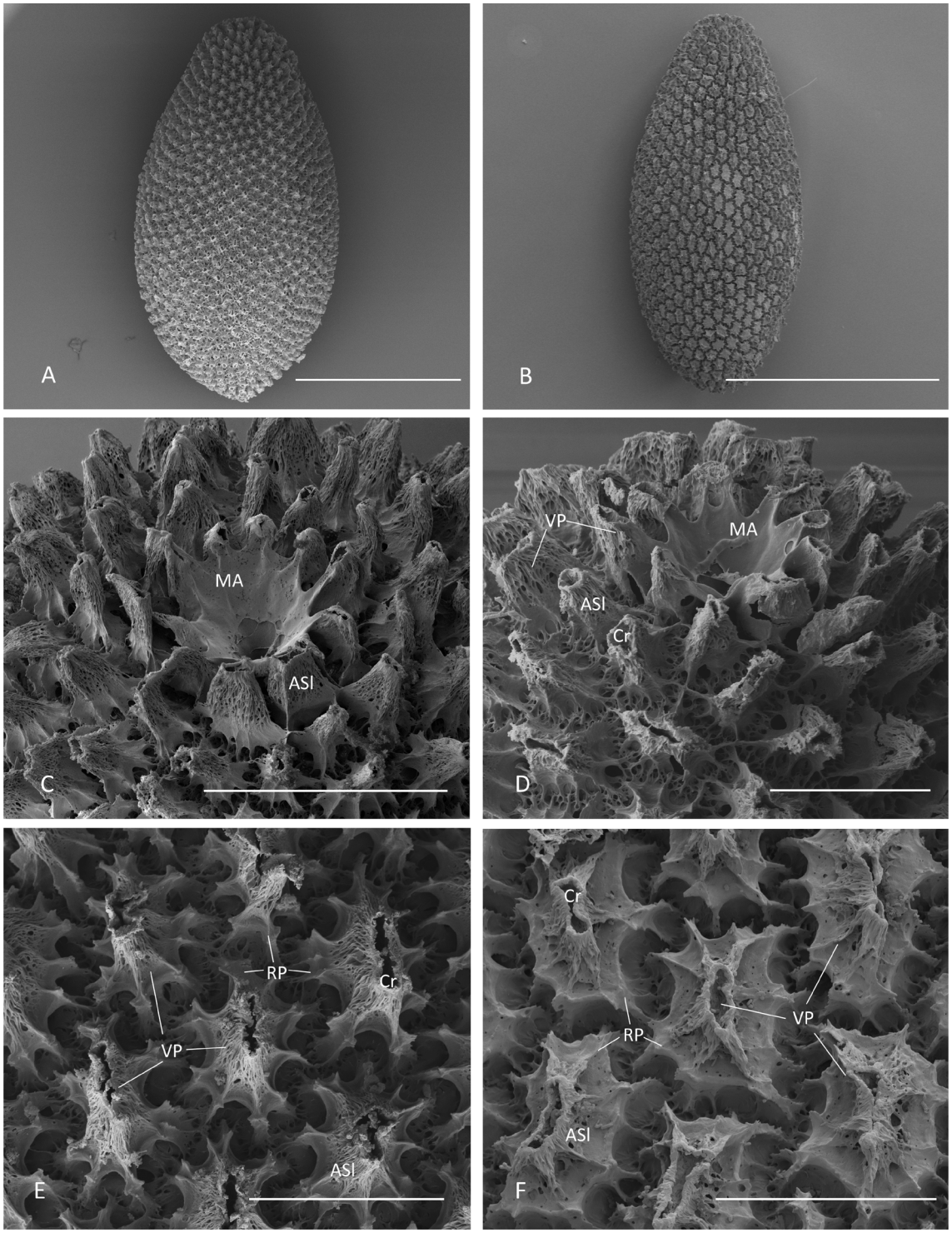

FIGURE 1. Egg of *M. myrmicae* (A, C, E) and *M.analis* (B, D, F): A, B—lateral view; C, D—detail of anterior pole with micropyle area. E, F—chorion microsculpture of volcano-like processes. A, B = 500 μm; C = 100 μm; D, E = 50 μm; F = 40 μm. ASl, alveolate slope; Cr, crater; MA, micropyle area; RP, radial projection; VP, volcano-like process.

Third instar larvae

Body features*.** Body strongly convex dorsally, nearly semi-circular in transverse section, ventral side ("foot") flat. ***Dorsal reticulation. Dorsal reticulation resulting from a peculiar net-like pattern of numerous processes, forming intersecting rows ("meshes"). Dorsal cuticular surface with granulate microsculpture. ***Posterior spiracular tuber-***

cle. Impair respiratory structure strongly sclerotised, emerging perpendicularly from posterodorsal part of abdomen. Surface of the posterior spiracular tubercle covered laterally with many polygonal plates; an apical furrow often present in the middle, dividing the apex into two halves. Apex of posterior spiracular tubercle with two round holes; apical spiracular plates smooth, with irregular margins, furrowed by numerous groups of narrow respiratory fissures, radially arranged. ***Marginal band.*** Fringe of elongated, parallel, radially projecting processes, surrounding the whole perimeter of the body except for the anteromedial furrow; processes not forming waives but showing double alternate conformation, apically bifurcate or single.

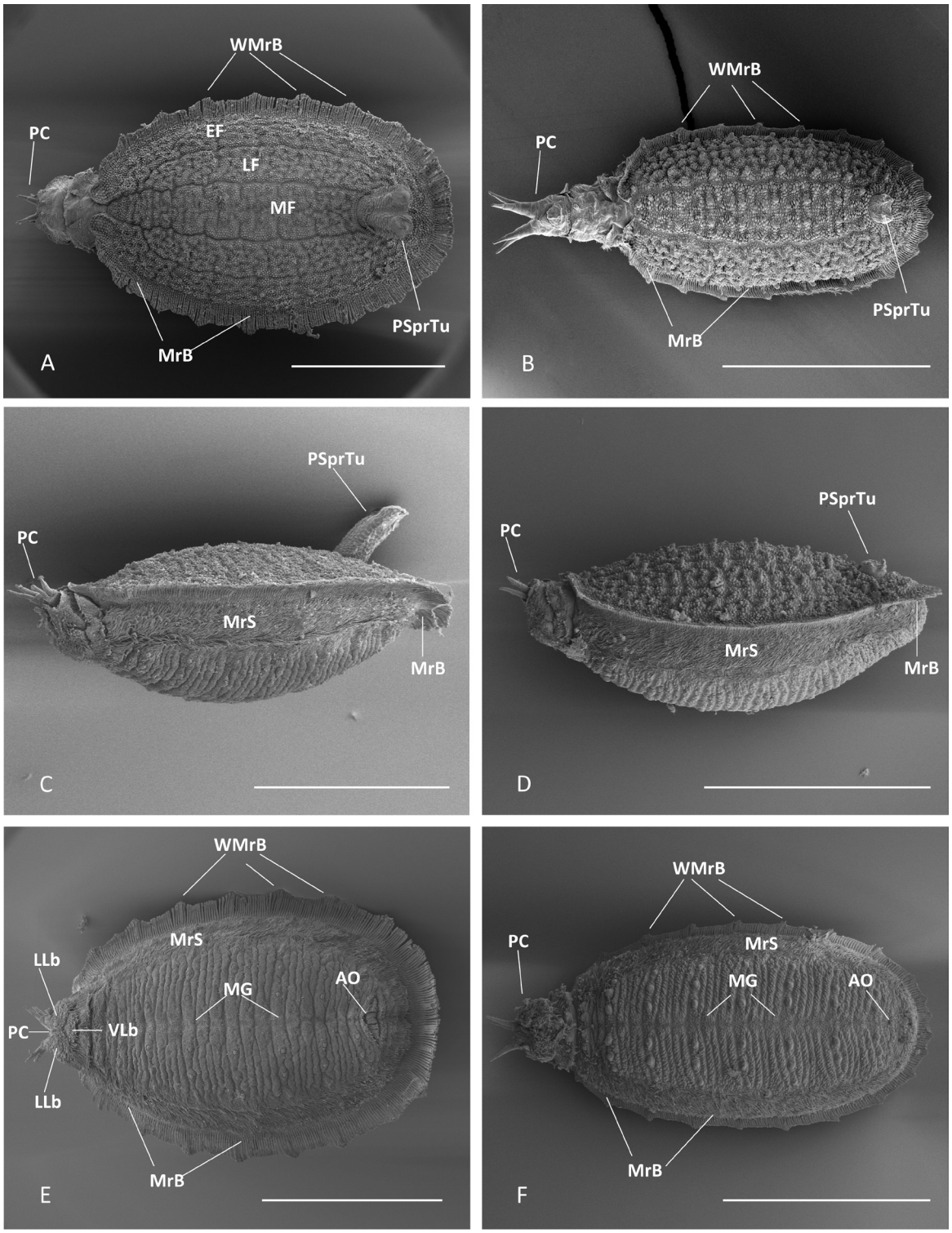

FIGURE 2. First instar larvae of *M. myrmicae* (A, C, E) and *M. analis* (B, D, F): A, B—dorsal view; C, D—lateral view; E, F—ventral view. A–F = 500 µm. AO, anal opening; LLb, lateral lobe; MrB, marginal band; MG, medial groove; MrS, marginal stripe; PC, pseudocephalon; PSprTu, posterior spiracular tubercle; VLb, venteral lobe; WMrB, waves of marginal band.

Puparia

Main features. Puparium very similar to the third instar larva except for the following characters: 1) body surface strongly sclerotised and reddish brown; 2) pseudocephalon retracted; 3) two anterodorsal spiracular tubercles emerging from the cuticular surface, showing variable shapes.

Immature stages of *Microdon myrmicae*

Egg

Figs 1, 11

Width = 587.4 ± 36.61 μm; length = 1.08 ± 0.03 mm (n = 10).

First instar larva

Figs 2−6, 11, Supplementary Material 1

Body width = 679.5 ± 55.42 μm; body length = 0.996 ± 0.08 mm (n = 10). ***Body features.*** Body with regularly rounded sides. Four longitudinal grooves present dorsally (Fig. 2A) dividing dorsal body surface into five main longitudinal fields: one medial, two lateral and two external fields. Medial field partially divided into two halves by a longitudinal, medial line. Dorsal surface with regularly spaced "flower-like" sensilla (Figs 3A, E): medial field with two longitudinal rows of nine sensilla; each lateral field with 13 sensilla arranged in two rows (seven along lateral groove and six along medial groove); each external field with one row of 10 sensilla. Each sensillum (Fig. 3E) composed of a cylindrical base, with many imbricate, thick sculpticels, apically with a medial flower-like structure with a variable number (5–10) of long lobes, pointed at tip, encircling a medial dome. Ventral surface covered medially by pointed microsculpture, finely pilose on sides. Ventral flower-like sensilla (Figs 3C, G) similar to dorsal ones except for flat, soft, unsculptured base and flat, thin, distinctly pointed lobes. ***Pseudocephalon.*** Two pairs of sensorial organs on dorsal surface of pseudocephalon (Figs 4E, G), one anterior (Fig. 4E) and one posterior (Fig. 4G), each composed of clusters of four short and one long trichoid sensilla emerging from bulbous, hollow base. ***Posterior spiracular tubercle.*** Impair respiratory structure, elongated in shape; surface of spiracular tubercle with peculiar microsculpture, completely covered by imbricate, sclerotised scales with an irregularly indented apex (Fig. 5A). Apex of tubercle with two circular smooth plates, slightly convex (Fig. 5C). ***Marginal band.*** Distinctly long fringe, length of processes regularly varying, showing eight waves on each side (Figs 2A, E, 6A, C, E). Each individual process composed of an elongate stem and an apical fringed brush (Figs 6A, C, E); the stem showing two very different surfaces: dorsal surface apparently articulated with 4–5 imbricated joints, the distal one fringed apically (Figs 6A, E); ventral surface completely smooth (Fig. 6C).

Third instar larva

Figs 7−9, 11

Body width = 4.5 ± 0.7 mm; body length = 6.1 ± 0.8 mm (n = 10). ***Dorsal reticulation.*** Dorsal reticulation reduced to a narrow, lateral belt around the perimeter of abdomen (Figs 7A, B). Each reticulation process showing sub circular groups of 5–9 umbrella-like structures with a flattened, circular apex (Figs 7A, B). ***Posterior spiracular tubercle.*** Dome shaped with two round holes spaced 1.2 times as long as their diameter; apex divided into two halves (Figs 8A, B) by a narrow furrow showing irregular plates. ***Marginal band.*** Processes on the marginal band short and thick, set close to one another, parallel, radially projecting, with suboval basal "joints", not imbricated, the last one produced into a flat medial brush. Processes on the marginal band of three types (Figs 9A, B): type one basally 4–jointed and apically single and flat, with medial brush 2–lobed; type two basally 3–jointed and apically bifurcate, with medial brush 1–lobed; type three basally 3–jointed and apically produced into a group of three spiniform setae. Type one and type two regularly alternating in sequence, type three irregularly present between two type one processes.

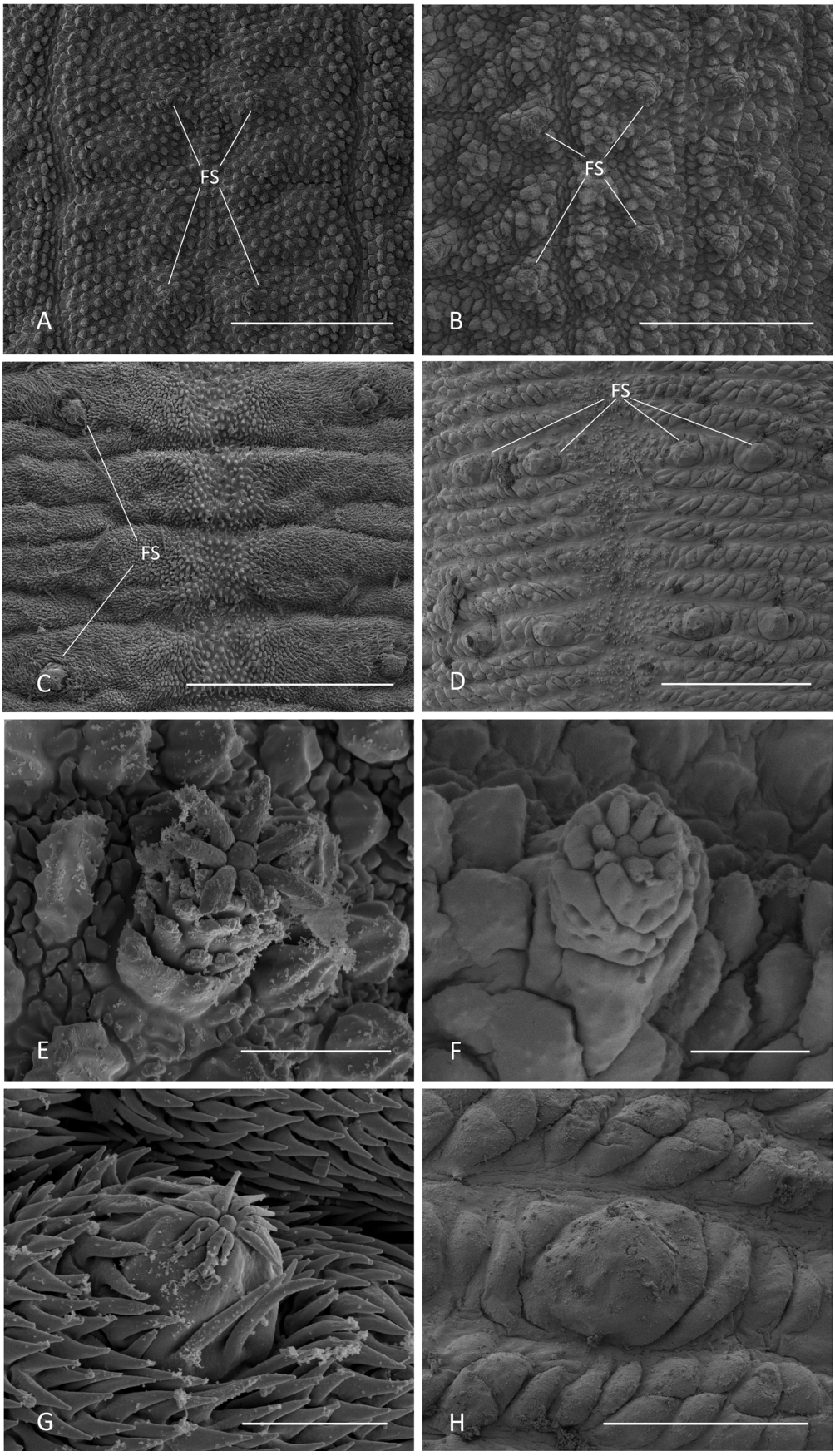

FIGURE 3. Details of first instar larvae of *M. myrmicae* (A, C, E, G) and *M. analis* (B, D, F, H): A, B—dorsal microsculpture; C, D—ventral microsculpture; E, F—dorsal flower–like sensilla; G, H—ventral flower like sensilla. A, B, C, D = 100 µm; E, F, G = 10 µm; H = 30 µm. FS, flower-like sensilla.

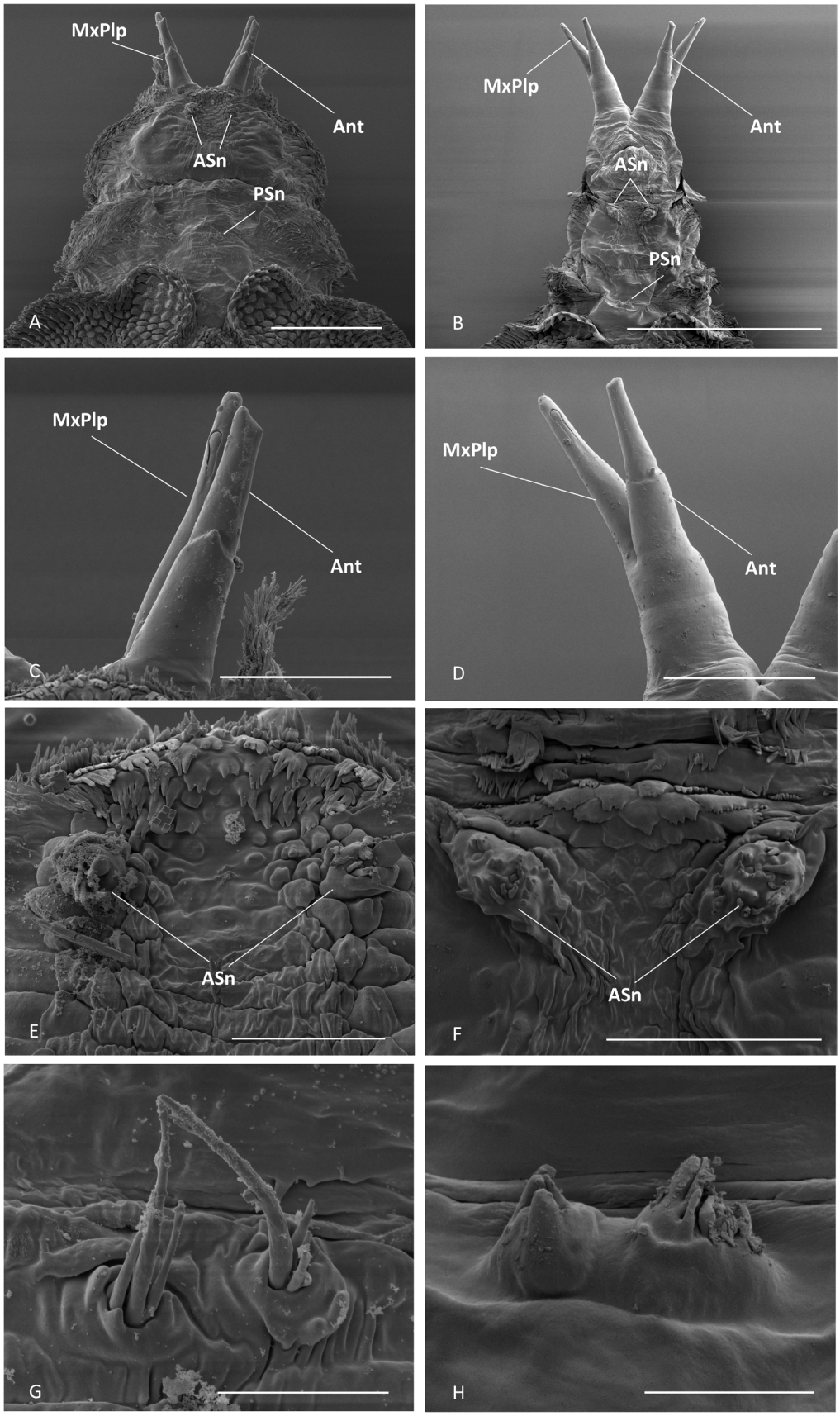

FIGURE 4. Pseudocephalon of first instar larvae of *M. myrmicae* (A, C, E, G) and *M. analis* (B, D, F, H): A, B—dorsal view; C, D antennomaxillary lobes; E, F—anterior sensorial organs; G, H—posterior sensorial organs. A = 100 µm; B = 200 µm; C = 40 µm; D = 50 µm; E, F = 30 µm; G, H = 10 µm. Ant, antenna; Asn, anterior sensillum; MxPlp, maxillary palp; Psn, posterior sensillum.

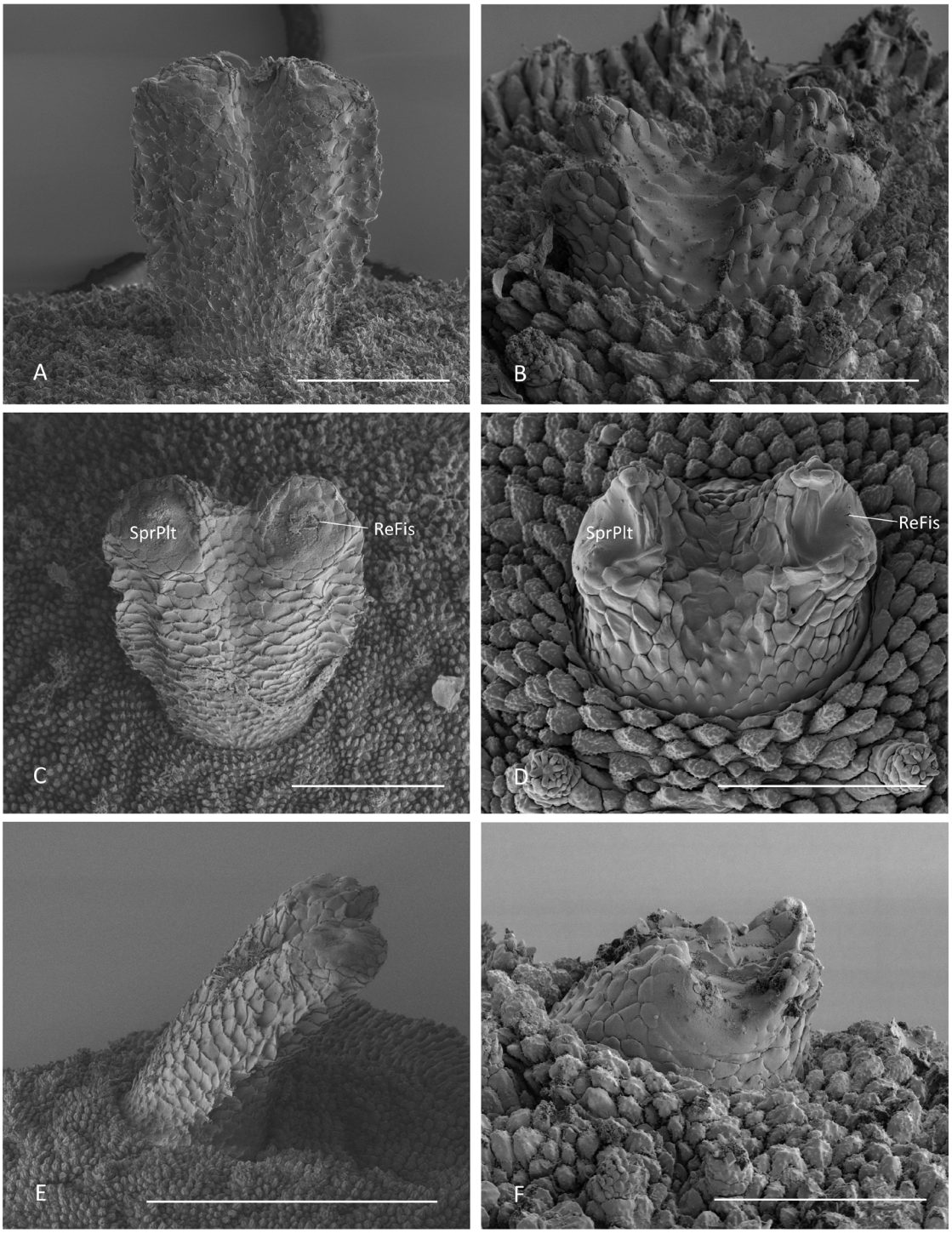

FIGURE 5. Posterior spiracular tubercle of first instar larvae of *M. myrmicae* (A, C, E) and *M. analis* (B, D, F): A, B—anterior view; C, D—apical view; E, F—lateral view. A, C = 100 µm; B–, D= 50 µm E = 200 µm. ReFis, respiratory fissures; SprPlt, spiracular plate.

Puparium

Figs 10, 11

Body width = 6.1 ± 0.5 mm; body length = 7.9 ± 0.7 mm (n = 10). ***Anterior spiracular tubercles.*** Length of each tubercle about 1.4 times as long as wide, conical, tapering at the apex (Fig. 10A, B), smooth at the base, with the apex furrowed by about 150 respiratory fissures (Figs 10A); each fissure laying on a small papilla (Fig. 10B).

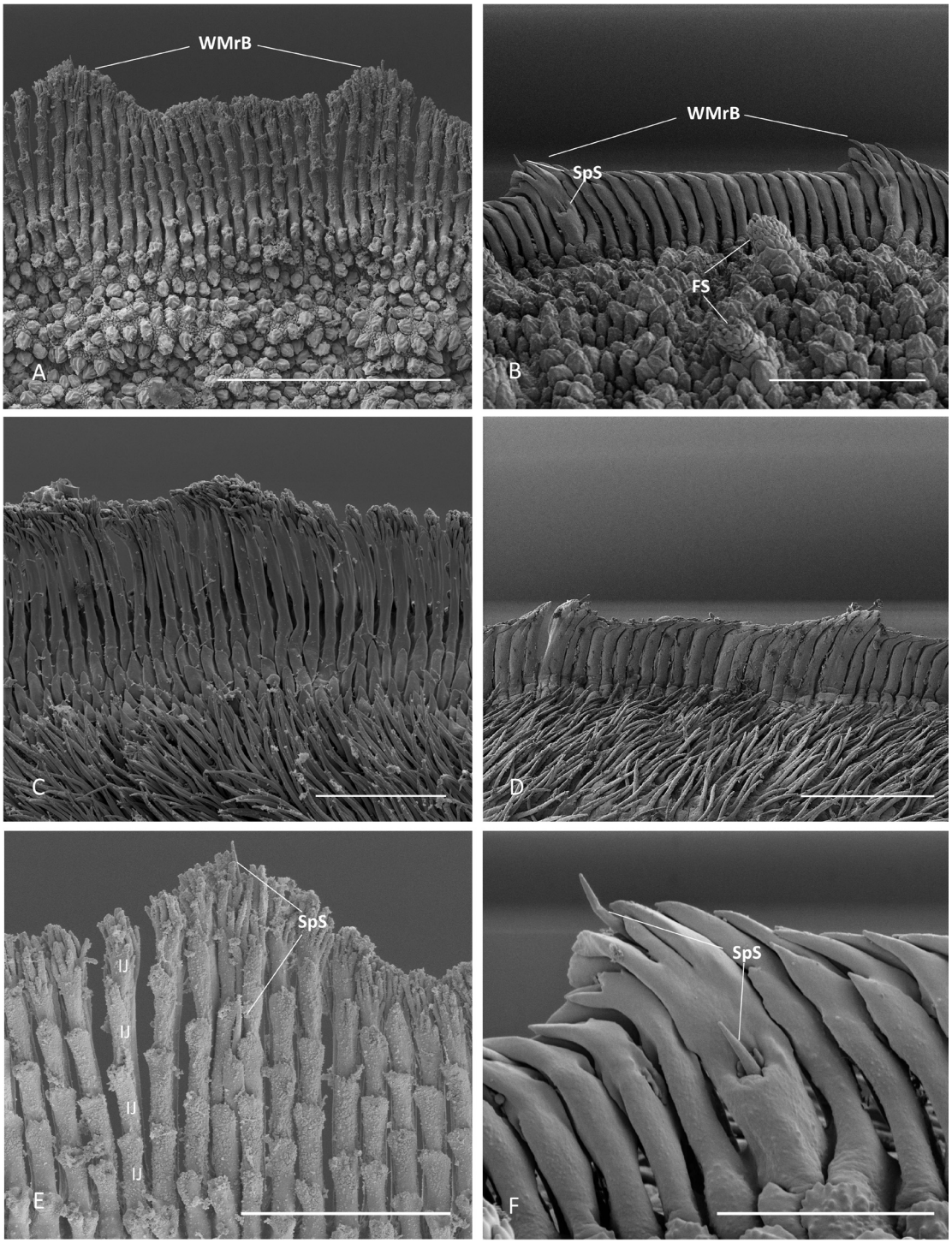

FIGURE 6. Marginal band of first instar larvae of *M. myrmicae* (A, C, E) and *M. analis* (B, D, F): A, B—dorsal view; C, D—ventral view; E, F—detail of processes, dorsal view. A = 100 µm; B, C, D = 50 µm; E = 40 µm; F = 20 µm. FS, flower–like sensilla; IJ, imbricate joint; SpS, spiniform setae; WMrB, waves of marginal band.

Immature stages of *Microdon analis*

Egg

Fig. 1

Width = 450.71 ± 52.89 µm; length = 897.41 ± 59.72 µm (n = 10).

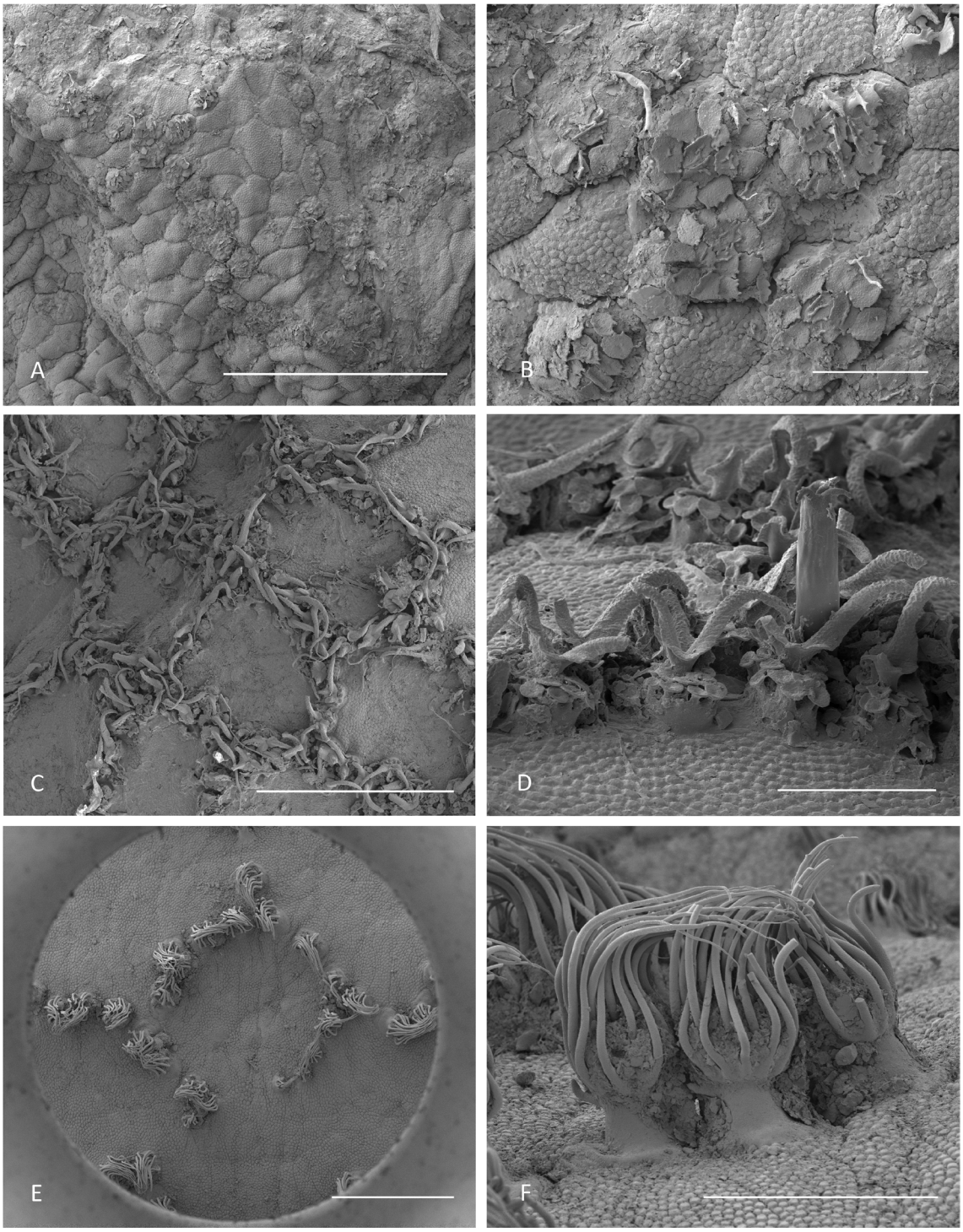

FIGURE 7. Dorsal reticulation of third instar larvae of *M. myrmicae* (A, B), *M. analis* (C, D) and *M. devius* (E, F); left, general view; right, detail of single reticulation processes. A = 400 μm; B = 50 μm; C, E = 500 μm; D = 100 μm; F = 200 μm.

First instar larva

Figs 2−6, Supplementary Material 2

Body width = 456.23 ± 29.41 μm; body length = 900.77 ± 75.14 μm (n = 10). ***Body features*.** Body rounded anteriorly and posteriorly, but subparallel on sides. Two longitudinal grooves present dorsally (Fig. 2B), dividing dorsal body surface into three main longitudinal fields: one medial, two external marginal fields. Dorsal surface adorned with raised sculpture and showing 62 regularly spaced flower-like sensilla (Fig. 3B) distributed as follows: medial

field with two longitudinal rows of nine sensilla; each marginal field with 22 sensilla. Each flower-like sensillum (Fig. 3F) composed of a long cylindrical base, apically with a medial flower-like structure with a variable number (6−7) of short lobes not exceeding the diameter of the base. Ventral surface without hairy microsculpture, showing transverse twisted protuberances (Fig. 3D). Ventral flower-like sensilla (Fig. 3H) dome-shaped, with an unsculptured simple base, without radial lobes. ***Pseudocephalon.*** Distal segment of the antenna distinctly narrowed at the apex (Figs 4B, D). Posterior sensorial organs on dorsal surface of pseudocephalon composed three short trichoid sensilla emerging from bulbous base (Fig. 4H). ***Posterior spiracular tubercle.*** Impair structure, short, bilobed. Surface of spiracular tubercle with microsculpture composed by thick, pointed, slightly imbricated scales (Figs 5B, D, F). Apex with depressed plates, laterally produced into four spikes: posterior spikes longer than the anterior (Figs 5D, F). ***Marginal band.*** Distinctly short fringe, showing 10 waves on each side (Figs 6B, D, F). Each simple process smooth both dorsally and ventrally (Figs 6B, D, F).

Third instar larva
Figs 7–9

Body width = 6.41 ± 0.64 mm; body length = 8.25 ± 0.54 mm (n = 10). ***Dorsal reticulation.*** Dorsal reticulation extended over the whole dorsal body surface. Each process divided into a dome-shaped basal disk supporting a branched spine (Figs 7C, D). Spines 2–branched not articulating with disk; processes disposed in 1–2 rows; trumpet-shaped tubercles (Fig. 7D) in 2–3 concentric circles on basal disk around spine base; dorsal spine branches intertwined extensively with branches originating 3–4 spines away. Dorsal flower-like sensilla with cylindrical smooth base and 3–4 lobes radiating from apex, lacking the central dome (Fig. 7D), always present at intersections of process rows and extending well above spines; each sensillum 4–5 times height of undivided spine base, 2–3 times length of processes. ***Posterior spiracular tubercle.*** Slightly wider than long structure, subrectangular both in dorsal and anterior views, concave medially (Figs 8C, D), with two round holes spaced 1.2 times as long as their diameter. Base of posterior spiracular tubercle encircled by a smooth cuticular crown. Spiracular plates flattened to slightly convex, of variable shape, separated by a broad and deep midsagittal cleft (Figs 8C, D). ***Marginal band.*** Processes on the marginal band apparently without basal articulated joints (Figs 9C, D).

Puparium
Figs 10, 11

Body width = 7.4 ± 0.4 mm; body length = 9.2 ± 0.3 mm (n = 10). ***Anterior spiracular tubercles.*** Length of each tubercle about three times as long as wide, subcylindrical, with sides entirely and strongly wrinkled, blunt at the apex, with the apex furrowed by about 30 respiratory fissures (Figs 10C, D). Each fissure laying on a small papilla (Fig. 10D).

Immature stages of *Microdon devius*

Third instar larva
Figs 7–9

Body width = 7.2 ± 0.6 mm; body length = 9.4 ± 0.9 mm (n = 10). ***Dorsal reticulation.*** Dorsal reticulation extended over the whole dorsal body surface. Each process resembling a sea anemone, being divided into a smooth, columnar trunk topped by a brush composed by 20–30 long, flexible filaments (Fig. 7F). ***Posterior spiracular tubercle.*** Longer than wide, subquadrate in dorsal view, concave medially, bulged sub–basally; distance between spiracular holes 1.8 times as long as their diameter (Figs 8E, F). The base of posterior spiracular tubercle encircled by a smooth cuticular crown (Figs 8E, F). Spiracular plates flattened to slightly concave, of variable shape, separated by a broad and deep midsagittal cleft. ***Marginal band.*** Processes on the marginal band apparently without basal articulated joints (Figs 9E, F). Processes on the marginal band of three types: type one long and single, apically fringed; type two short and bifurcate, apically 2–lobed; type three composed by two type one flanking a cluster of three spiniform setae (Figs 9E, F).

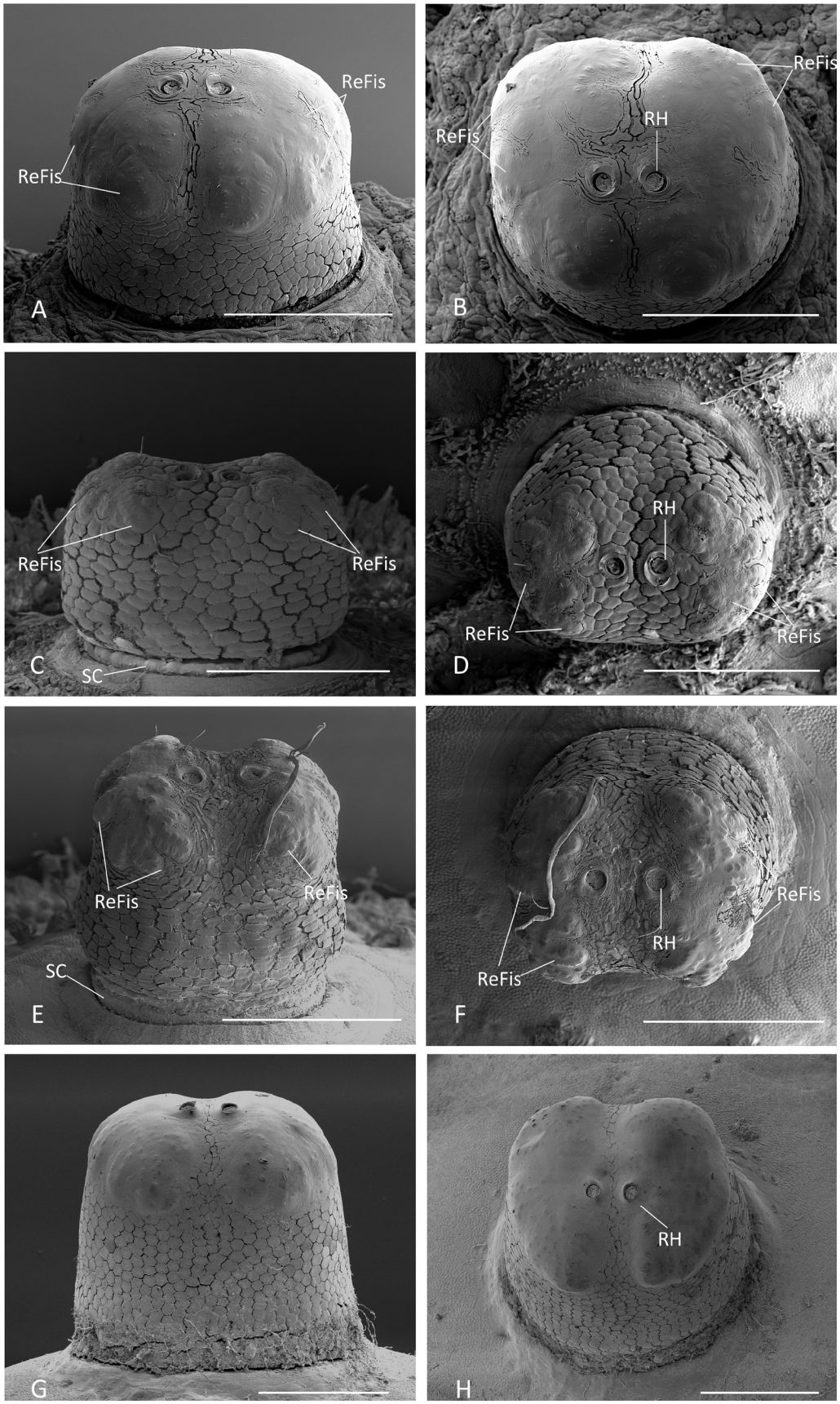

FIGURE 8. Posterior spiracular tubercle of third instar larvae of *M. myrmicae* (A, B), *M. analis*(C, D), *M. devius* (E, F) and *M. mutabilis* (G, H); left, anterior view; right, apical view. A–H = 500 μm. ReFis, respiratory fissures; RH, respiratory hole; SC, smooth crown.

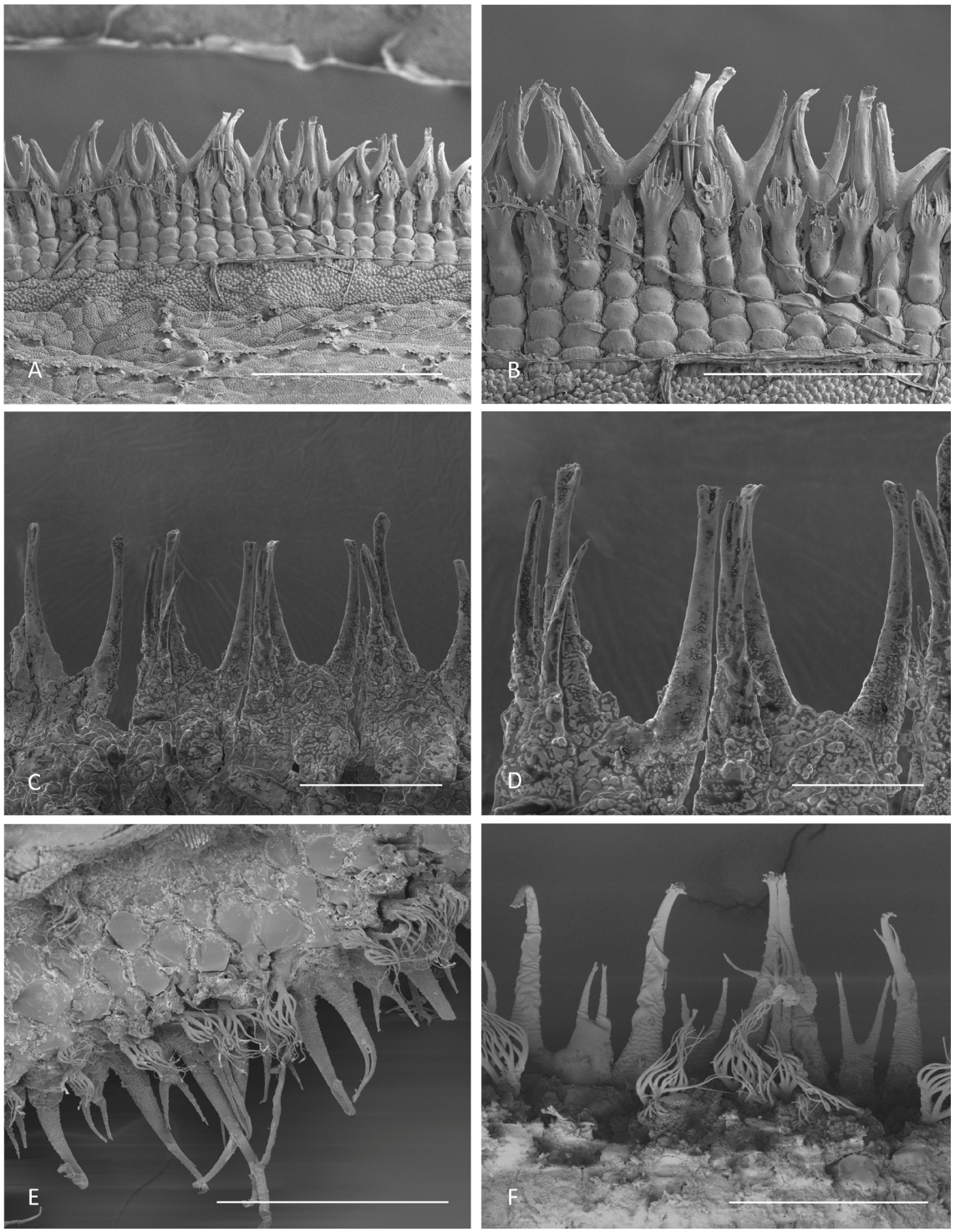

FIGURE 9. Marginal band of third instar larvae of *M. myrmicae* (A, B), *M. analis* (C, D) and *M. devius* (E, F); left, dorsal view; right, detail of marginal band. A = 500 μm; B, E = 300 μm; C = 100 μm; D = 50 μm; F = 200 μm.

Puparium

Figs 10, 11

Body width = 7.3 ± 0.4 mm; body length = 9.7 ± 0.5 mm (n = 10). ***Anterior spiracular tubercles.*** Length of each tubercle 2.25 times as long as wide and strongly curved (Fig. 11F), subcylindrical, slender, somewhat pointed at the apex, entirely furrowed by about 180 respiratory fissures (Figs 10E, F). Each fissure laying on a small papilla (Fig. 10F).

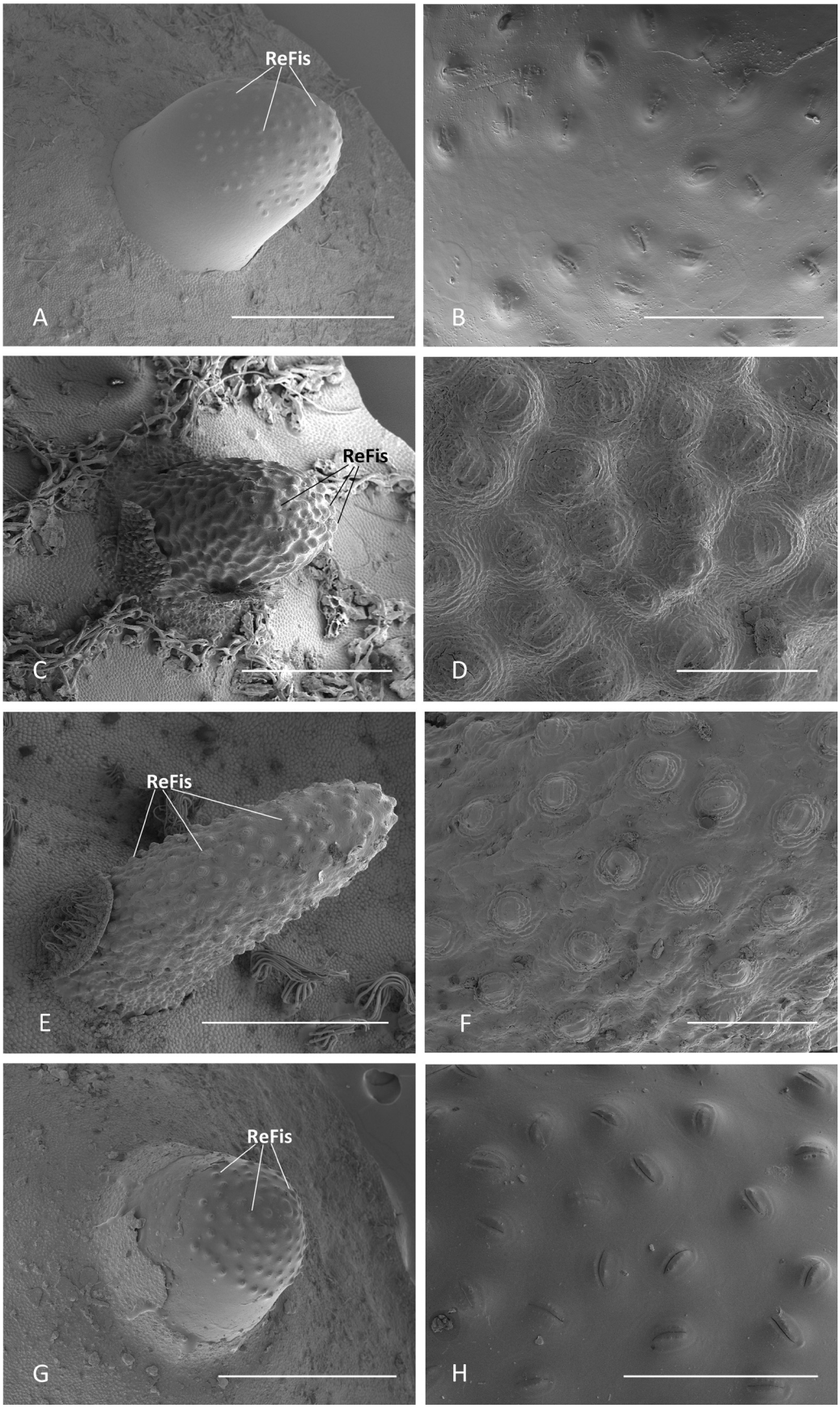

FIGURE 10. Anterior spiracular tubercles of puparia of *M. myrmicae* (A, B), *M. analis*(C, D), *M. devius* (E, F) and *M. mutabilis* (G, H); left, lateral view; right, detail of respiratory fissures. A, E, G = 500 μm; B, F, H = 100 μm; C = 400 μm; D = 50 μm. ReFis, respiratory fissures.

FIGURE 11. Eggs of *M. myrmicae* trapping small drops of water (pointed with red arrows) with their volcano-like processes (A); third instar *M. myrmicae* larvae with larvae and workers of *Myrmica scabrinodis* (B); third instar larva and first instar larva (red arrow) of *M. myrmicae* with *M. scabrinodis* larvae, ventral view (C); *M. myrmicae* adult emerging from puparium, anterior view (D); two *M. analis* pupae and a puparium (E); *M. devius* pupa (F).

Keys to immature stages of the European species of *Microdon*

First instar larvae

1 Body about 1.5 times as long as wide; posterior spiracular tubercle long (more than 1.5 times as long as wide at base); marginal band elongate, showing eight waves on each side . . . . . *M. myrmicae*/*M. mutabilis*

- Body about two times as long as wide; posterior spiracular tubercle very short (wider than long at base); marginal band short, showing 10 waves on each side . . . . . *M. analis*

Puparia

1 Most of the dorsal surface of puparium smooth and bare, with reticulation reduced to a narrow, lateral belt 2
- Dorsal surface of puparium completely covered by a dorsal reticulation 3

2 Anterior spiracular tubercles conical, tapering at apex; anterior spiracular tubercles about 1.4 times as long as wide at base; each reticulation process composed of groups of umbrella-like structures . . . . . *M. myrmicae*
- Anterior spiracular tubercles dome-shaped, blunt at apex; anterior spiracular tubercles about 0.8 times as long as wide at base; each reticulation process composed of stringy, extended projections . . . . . *M. mutabilis*

3 Meshes of the dorsal reticulation no broader than the basal diameter of the posterior spiracular tubercle; posterior spiracular tubercle wider than long; anterior spiracular tubercles straight 4
- Meshes of the dorsal reticulation two times as broad as the basal diameter of the posterior spiracular tubercle; posterior spiracular tubercle longer than wide; anterior spilacural tubercles laterally curved . . . . . *M. devius*

4 Anterior spiracular tubercles equal or more than two times as long as wide 5
- Anterior spiracular tubercles one time only as long as wide, or shorter than wide . . . . . *M. miki*

5 Anterior spiracular tubercles nearly cylindrical, about three times as long as wide; posterior spiracular tubercle light-brown contrasting with the reddish-brown apical spiracular plates . . . . . .*M. analis*
- Anterior spiracular tubercles clearly conical, about two times as long as wide; posterior spiracular tubercle uniformly reddish-brown . . . . . *M. major*

Locomotion of first instar larvae in *M. analis* and *M. myrmicae*

Supplementary Material 1, 2

Movement of first instar larva is mainly performed by using the ventral muscular plate (resulting from fusion between thoracic and abdominal sternum). The movement (postero-anterior waves of contraction and expansion that sweep along the body) is mainly localized on the ventral side ("foot") but can involve the whole body in first instar larvae, which can at times rapidly or slowly stretch and contract their bodies. The larva can also move backwards and sideways, or just rotate its body by contracting and partially folding its sides to change direction. For backward and sideways movements, the waves of contraction start on the anterior part of the plate and go backwards. In contrast to the third instar larva, the mandibles of first instar larvae are involved in locomotion and hook on to the substrate to establish an anchor point. The marginal band apparently does not play a role during locomotion.

Discussion

Broadly, the eggs, larvae and puparia of all three species here described (*Microdon analis, M. devius, M. mymricae*) and *M. mutabilis* (Scarparo *et al.* 2017) are similar in shape and appearance. We supply a general description of immature stages valid for all *Microdon* species, as far as is known. However, there are considerable differences between species in the detail of certain key morphological structures. Here we compare and contrast the following features: dorsal reticulation, marginal band, flower–like sensilla, posterior spiracular tubercles and anterior spiracular tubercles. Individually or collectively these characters allow all four species to be told apart as immatures.

Eggs. The eggs of all the species which have been examined so far, in both Europe and North America, do not show any significant differences: those of the three species described here for the first time are similar to those of *M. mutabilis* (Scarparo *et al.* 2017) and to those of four Nearctic species described by Garnett *et al.* (1990). A major shared character is the presence of volcano-like processes, covering the entire chorion of the egg. Our observations made in the lab suggest that these peculiar structures may have an important function preventing egg dehydration, trapping small drops of water within their apical depressions or "craters" (see Fig. 11A). However direct field observations and lab experiments of desiccation are needed to confirm this hypothesis.

First instar larva morphology. The first instar larvae of *M. analis* and *M. myrmicae* are markedly different. The long posterior spiracular tubercle, a major distinctive feature in *M. myrmicae* and *M. mutabilis* larvae (Scarparo *et al.* 2017), is much reduced in *M. analis* to a small and short posterior protuberance which is not readily recognizable from the dorsal microsculpture. Thus, it seems unlikely that this structure could act as a handle that the ants

can grab to transport larvae into the nest, as was hypothesized by Scarparo *et al.* (2017) in *M. mutabilis.* Rather, the reduced size could prevent abrasion damage from rubbing against nest walls: the much reduced posterior spiracular tubercle in *M. analis* larvae may reflect the very different habitat the species occupies compared to that of *M. myrmicae*. While *M. myrmicae* first instar larvae inhabit wet underground ant nests where a long snorkel can be helpful to breathe, *M. analis* larvae live mainly in nests built under the bark of dead logs where a short spiracular tubercle may minimize abrasion against the hard wood that forms the nest walls.

The marginal band, with long processes, has in *M. myrmicae* a double microsulpture, dorsally with imbricated joints and ventrally entirely smooth, whereas the short *M. analis* marginal band is unsculptured on both sides. However, both species show apical and subapical spiniform setae on the edges of the marginal band. Additional observations in the lab confirmed (Scarparo *et al.* 2017) that the marginal band is not involved in the locomotory action.

The dorsal flower-like sensilla are rather similar in both species, although those of *M. analis* are longer and with shorter "petals" than those of *M. myrmicae.* A completely different microsculpture was found in *M. analis*'s ventral flower-like sensilla, the flower-like shape being completely lost and replaced by a simple dome–shaped structure. Compared to the few other known first instar larvae from North American species like *M. albicomatus* Novak, *M. piperi* Knab*, M. xanthopilis* Townsend (Garnett *et al.* 1990) and *M. cothurnatus* Bigot (Akre *et al.* 1973), it seems that *M. analis* is very similar to these, sharing a short posterior spiracular tubercle, marginal band processes lacking segmentation, and long dorsal flower-like sensilla. Unfortunately, we did not succeed in rearing any *M. devius* first instar larvae

Third instar larva morphology. The main difference between the three analysed species lies in the dorsal reticulation, highly reduced in *M. myrmicae* to a dorsolateral band and well developed in *M. analis* and *M. devius* covering the entire dorsal surface. Even the individual dorsal reticulation processes appear very different, resembling sea anemones in *M. devius*, or simple projections with trumpet-shaped tubercles at the base in *M. analis,* while in *M. myrmicae,* umbrella-like structure, lacking any kind of projections are present.

The shape of the posterior spiracular tubercle is helpful in the identification of the three species: short and subrectangular in *M. analis;* longer than wide, subquadrate and bulging sub-basally in *M. devius*; dome-shaped in *M. myrmicae*.

The third important character, difficult to observe in detail using light microscopy, is the marginal band. Although the double alternate conformation, apically bifurcate or single, and groups of spiniform setae persists in all three species, SEM reveals several minor differences. For example, the presence of basal articulated joints is only present in *M. myrmicae* and *M. mutabilis* but is lacking in *M. analis* and *M. devius*

Puparium morphology. In general, puparia of this genus strongly resemble the third larval instar, except for two evident features: the brown–reddish colour of the body, a sign of a high sclerotization, and the presence of two anterior spiracular tubercles (also known as prothoracic horns or anterior respiratory horns) that emerge a few hours after pupation. These spiracular tubercles are respiratory organs that are important diagnostic tools to discern cryptic species. In fact, the major diagnostic feature to distinguish between *M. mutabilis* and *M. myrmicae* is the length of prothoracic horns, those of *M. myrmicae* being longer than those of *M. mutabilis* (Schönrogge *et al.* 2002). Furthermore, we found a different shape to these anterior spiracular tubercles, conical and tapered at the apex in *M. myrmicae* but dome–shaped in *M. mutabilis*. By comparison in both *M. analis* and *M. devius* the anterior spiracular tubercles are subcylindrical, those of *M. analis* being furrowed by respiratory fissures only at the apex, as in *M. mutabilis* and *M. myrmicae*, those in *M. devius* having fissures are scattered over the entire surface of the spiracle.

***Differences between* M. mutabilis *and* M. myrmicae.** *Microdon myrmicae* was recently described by Schönrogge *et al.* (2002) who split *Microdon mutabilis* into two species: *Microdon myrmicae*, a parasite of the ant genus *Myrmica*, and *Microdon mutabilis*, a parasite of *Formica* species. These *Microdon* species are highly related phylogenetically to one another, even though they exploit hosts of two distant ant subfamilies (Formicinae and Myrmicinae). This case study outlines the considerable parasitic plasticity within *Microdon* species. The adults of these species are extremely similar, with no significant morphological differences (Schönrogge *et al.* 2002). This may reflect a recent evolutionary split due to host shift caused by potential local rarefaction/extinction of the primary host. Identification is based only on the characters of the pre–imaginal instars, like the length of anterior spiracular tubercles on the puparium. Here, we supply new morphological features which support the specific validity of *M. myrmicae*. We detected long hairs scattered on the dorsum of *M. myrmicae* puparia, while *M. mutabilis* is hairless. The presence of dorsal hairs could be related with the different microhabitats were these two species are found. *M. mutabilis* is recorded in consistently drier areas in comparison to the *M. myrmicae* sites, of which the optimal habitat

seems to be waterlogged areas dominated by *Juncus* spp., *Sphagnum* spp. (Schönrogge *et al.* 2002) and *Molinia caerulea* (Wolton 2011). The long hairs of *M. myrmicae* may create a gap between the puparium and the nest surface that permits a degree of air flow, possibly slowing down the development of potentially deadly moulds in the high humidity conditions that prevail in the nests of their host ants. *M. mutabilis* is known to have a more marked dorsal reticulation than *M. myrmicae* (Schönrogge *et al.* 2002) but the precise structural differences were previously unknown. We observed the dorsal reticulation of *M. myrmicae* to be much reduced and modified into groups of umbrella-like structure, lacking the stringy and extended projections typical of the dorsal reticulation processes of *M. mutabilis* (Scarparo *et al.* 2017).

Phylogenetic considerations. *M. analis* and *M. devius* third instar larvae and puparia share features which suggest a close phylogenetic affinity: extended dorsal reticulation, a wrinkled anterior spiracular tubercle, similar–shaped posterior spiracular tubercles and the same host genus. However, the shape of single dorsal reticulation processes suggests that *M. analis* is more closely related to *M. mutabilis* than to *M. devius*: while these processes are similar in *M. analis* and *M. mutabilis,* they are highly modified in *M. devius* resembling sea anemones. Also, *M. analis* anterior spiracular tubercles have respiratory fissures only at the apex, as in *M. myrmicae* and *M. mutabilis,* while they cover the entire structure in *M. devius.* However, no clear hypothesis can as yet be constructed as to the phylogenetic relationship among *Microdon* species based on immature morphological characters, since we are far from knowing the morphological diversity of the preimaginal stages of this genus.

Although numerous studies have been carried out to gain a better comprehension of Syrphidae and Microdontinae evolution (Rotheray & Gilbert 1999; Ståhls *et al.* 2003; Reemer 2013; Reemer & Ståhls 2013b; Young *et al.* 2016), *Microdon,* and indeed Microdontinae, taxonomy and phylogeny remains obscure, strongly hampered by numerous cryptic species and lack of studies on larvae and puparia.

At least in certain cases, the morphological variability among different species of *Microdon* is higher at the larval or puparial stage than at the adult stage, leading us to believe that the characters of immature stages may be very informative about phylogenetic relationships. This is especially true if studied using high resolution techniques such as scanning electron microscopy which can acquire information at an amazing level of detail. Structures such as the marginal band, dorsal reticulation, anterior spiracular tubercles, posterior spiracle, flower–like sensilla and other features could represent valid morphological characters for the comprehension of *Microdon* phylogeny, alone or in combination with adult features and with molecular analyses. Furthermore, SEM microscopy is a powerful tool for the resolution of *Microdon* taxonomy made uncertain by the presence of cryptic species. They could give exciting hints on the adaptive morphological functions in enabling the fly eggs, larvae and puparia to survive within the nests of their ant hosts, as predators of their larvae.

We hope that in future more syrphid researchers will dedicate effort to expanding knowledge of immature stages, contributing not only to better understanding of the taxonomy and systematics of Microdontinae, but also to our understanding of the evolution of *Microdon* host-parasite interactions.

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Author Contributions: GS, ADG designed the research; GS, RW, MM, LCP, ADG carried out field sampling; GS performed lab breeding; GS, ADG prepared specimens and acquired all images; GS, RW, ADG wrote the manuscript; GS, RW, MM, LCP, ADG revised the final manuscript.

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Captions of Supplementary Materials

Supplementary Material 1 Locomotion of first instar larva of *Microdon myrmicae.*
Supplementary Material 2 Locomotion of first instar larva of *Microdon analis.*