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The smallest insects evolve anucleate neurons

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ABSTRACT

The smallest insects are comparable in size to unicellular organisms. Thus, their size affects their structure not only at the organ level, but also at the cellular level. Here we report the first finding of animals with an almost entirely anucleate nervous system. Adults of the smallest flying insects of the parasitic wasp genus *Megaphragma* (Hymenoptera: Trichogrammatidae) have only 339–372 nuclei in the central nervous system, i.e., their ganglia, including the brain, consist almost exclusively of processes of neurons. In contrast, their pupae have ganglia more typical of other insects, with about 7400 nuclei in the central nervous system. During the final phases of pupal development, most neuronal cell bodies lyse. As adults, these insects have many fewer nucleated neurons, a small number of cell bodies in different stages of lysis, and about 7000 anucleate cells. Although most neurons lack nuclei, these insects exhibit many important behaviors, including flight and searching for hosts.

1. Introduction

Miniaturization is an evolutionary decrease in body size, an important trend in the evolution of animals (Hanken and Wake, 1993). Insects are one of the most successful at this evolutionary direction and a lot of them demonstrate extreme miniaturization. Size strongly determines the morphology, physiology, and biology of species (Schmidt-Nielsen, 1984). Structural trends associated with miniaturization have been described in many animals (Hanken and Wake, 1993). For vertebrates, e.g. Amphibia, they affect mainly particular aspects of anatomy of the skeleton (Hanken, 1983, 1985; Yeh, 2002). For some invertebrates miniaturization affect whole organs and organ system (Hanken and Wake, 1993). For small insects, considerable modifications have been described affecting almost all the organs (Grebennikov and Beutel, 2002; Polilov, 2005, 2008; Polilov and Beutel, 2010). In the smallest insects whose size is comparable to that of unicellular organisms (Fig. 1), miniaturization creates problems not only at the organ, but also at the cellular level (Rensch, 1948; Polilov and Beutel, 2009).

The nervous system of very small insects is of special interest because the neuronal control of behaviors depends in part on neuronal number (Kaas, 2000). In spite of their extremely small size, the smallest beetles of the family Ptiliidae (Coleoptera) have about 40,000 cells in their nervous systems (Polilov, 2008). These beetles have evolved a nervous system with considerably larger relative volume and considerably smaller cell size compared to those in larger members of related taxa. This decrease in cell size is accompanied by a considerable increase in the relative volume of the cytoplasm and an increase in the degree of chromatin compaction (Polilov, 2008). Similar evolutionary changes are observed in other very small insects.

The smallest flying insects belong to the genus *Megaphragma* (Hymenoptera: Trichogrammatidae). Their adult body length is only 170 μm in *Megaphragma caribea* and 200 μm in *Megaphragma mymaripenne*. The anatomy of these insects is of considerable interest for studies of the influence of size on tissue structure. Our initial anatomical study of *M. mymaripenne* revealed that their adult nervous system contained only a few hundred neuronal nuclei. This finding raised the question of whether the central nervous system of this species actually contains so few neurons, or something happens to their nuclei.

2. Materials and methods

2.1. List of taxa examined

M. mymaripenne Timberlake, 1924 (Hymenoptera: Trichogrammatidae), adults and pupae, collected in Blanes, Spain (2008) and Funchal, Madeira, Portugal (2009) were studied. For comparative purposes, specimens of adults and pupae of *Trichogramma evanescens* Westwood, 1833 were studied. The *Trichogramma* was obtained from LTD Centre Biotechnique, Khlebodarskoe, Ukraine.

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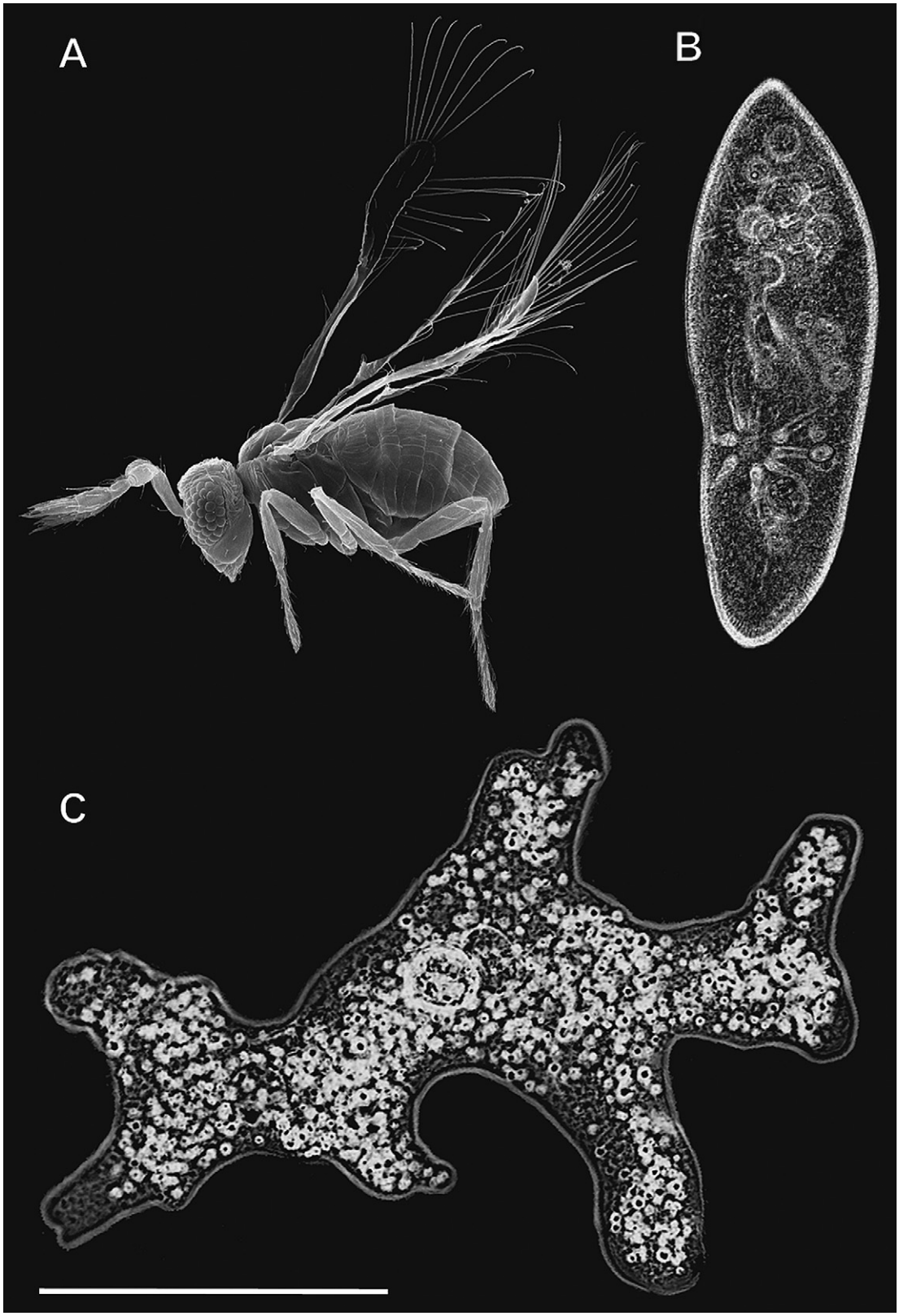

Fig. 1. Size of the smallest insect and two protozoans in comparison. (A) *Megaphragma mymaripenne*. (B) *Paramecium caudatum*. (C) *Amoeba proteus*. Scale bar for A–C is 200 μm.

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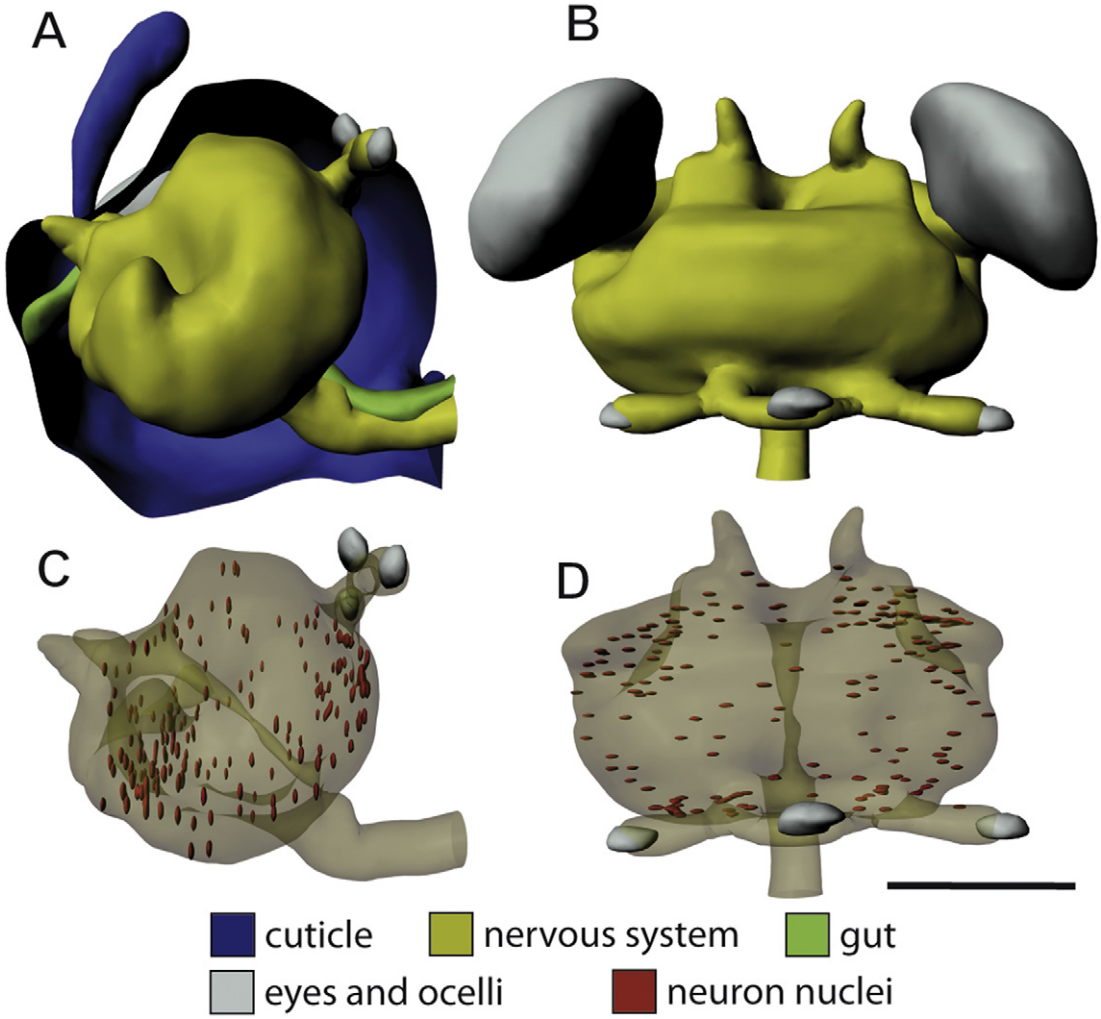

Fig. 2. Cephalic structure in *M. mymaripenne*, 3D-reconstruction. (A) Head capsule, CNS and gut; lateral view. (B) CNS, eyes and ocelli; dorsal view. (C, D) Brain (transparent) with sporadic neuron nuclei; lateral view (C) and dorsal view (D). Scale bar for A–D is 25 μm.

2.2. Histology

Adults and pupae were fixed in formaldehyde–ethanol–acetic acid (FAE). They were embedded in Araldite M, cut at 1 μm with a Leica microtome (RM 2255), and stained with toluidine blue. The serial sections were photographed with a AxioCam digital camera on a Zeiss Axioskop.

2.3. Three-dimensional (3D) reconstruction

A series of sequential cross-sections were used for 3D reconstructions. Based on the aligned image stacks, structures of adults and pupae were reconstructed with Bitplane Imaris. The volume of organs and the body (without legs, wings, antennae and pedipalps) was calculated based on 3D models using the Imaris statistics module.

2.4. Determining the number of neurons

In adult *Megaphragma* all neurons were counted in aligned image stacks. In pupal *Megaphragma* and in adult and pupal *Trichogramma* the number of neurons was estimated by determining the average linear size of a neuron cell body using the Fiala Reconstruct software, based on histological sections, and then calculating the number of neurons, taking into account cortex layer volume and average neuron cell body volume.

2.5. Scanning electron microscopy (SEM)

After cleaning, the specimens were critical point dried (Hitachi HCP-1) and coated with gold (Hitachi IB-3). Pictures were taken with a Jeol JSM-6380.

2.6. Transmission electron microscopy (TEM)

Material was fixed in 2% glutaraldehyde solution on 0.1 M cacodilat buffer pH 7.2 and post-fixed with 1% osmium tetroxide in the same buffer. Specimens were embedded in Epon 812, cut with LKB ultramicrotomes, stained with lead citrate, and examined with a Jeol JEM-1011 transmission electron microscope.

3. Results

A detailed study of the nervous system based on serial histological sections using 3D computer modeling and transmission electron microscopy shows that the central nervous system of adult *M. mymaripenne* displays considerable oligomerisation and concentration of ganglia, with the thoracic ganglia merged into a single

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synganglion positioned mostly in the metathorax, and the abdominal ganglia merged into a single synganglion. The brain of *M. mymaripenne* occupies a large part of the space within the head capsule (Fig. 2). The brain and other ganglia consist almost exclusively of neuropil (Fig. 3A–D) similar in appearance to that of larger insects.

The central nervous system contains only 339–372 ($M = 361$, $n = 3$) nuclei, with 179–253 ($M = 215$, $n = 3$) of them in the brain (Fig. 2C, D). These numbers are extremely low compared to adults of larger wasps in the genus *Trichogramma* of the same family that have about 37,000 nucleated neurons in the brain. The central nervous system of *M. mymaripenne* occupies 6% of the body volume, 2.9% by the brain. This ratio is markedly different form larger hymenopterans (e.g. *Apis*, honeybees) in which the brain occupies 0.35%–1.02% of body volume and 0.57% in ants of the genus *Formica* (Strausfeld, 1976; Wigglesworth, 1953) and 3.2% in Chalcidoidea, a parasitic wasp of the genus *Hemiptarsenus*.

The central nervous system of the pupae of *M. mymaripenne* has about 7199–7593 ($M = 7396$, $n = 2$) cells and a structure typical of insects at this developmental stage, i.e., every ganglion consists of neuropil surrounded by a cortex layer of neuron cell bodies (Fig. 3E–H). The central nervous system occupies 19% of the body volume in pupae, 11% by the brain, which contains about 4600 cells. During the final phases of pupal development, mass lysis of neuron bodies is observed. Since the number of neurons in late pupae and adults of the closely related *T. evanescens* remains almost unchanged, we assume that the number of cells in CNS in adult *Megaphragma* is the same as in pupae.

Lysis of nuclei and cell bodies in over 95% of the neurons is observed after the formation of the adult nervous system, before the adult emerges from the pupa. This high degree of lysis markedly decreases the absolute and relative volume of the nervous system, especially in the brain, which has a volume of 93,600 μm^3 in the pupa and only 52,200 μm^3 in the adult. This change in brain volume is accompanied by a peculiar transformation of the head capsule during later stages of pupal development. The head capsule of the pupa is initially considerably larger than that of adults due to a strongly

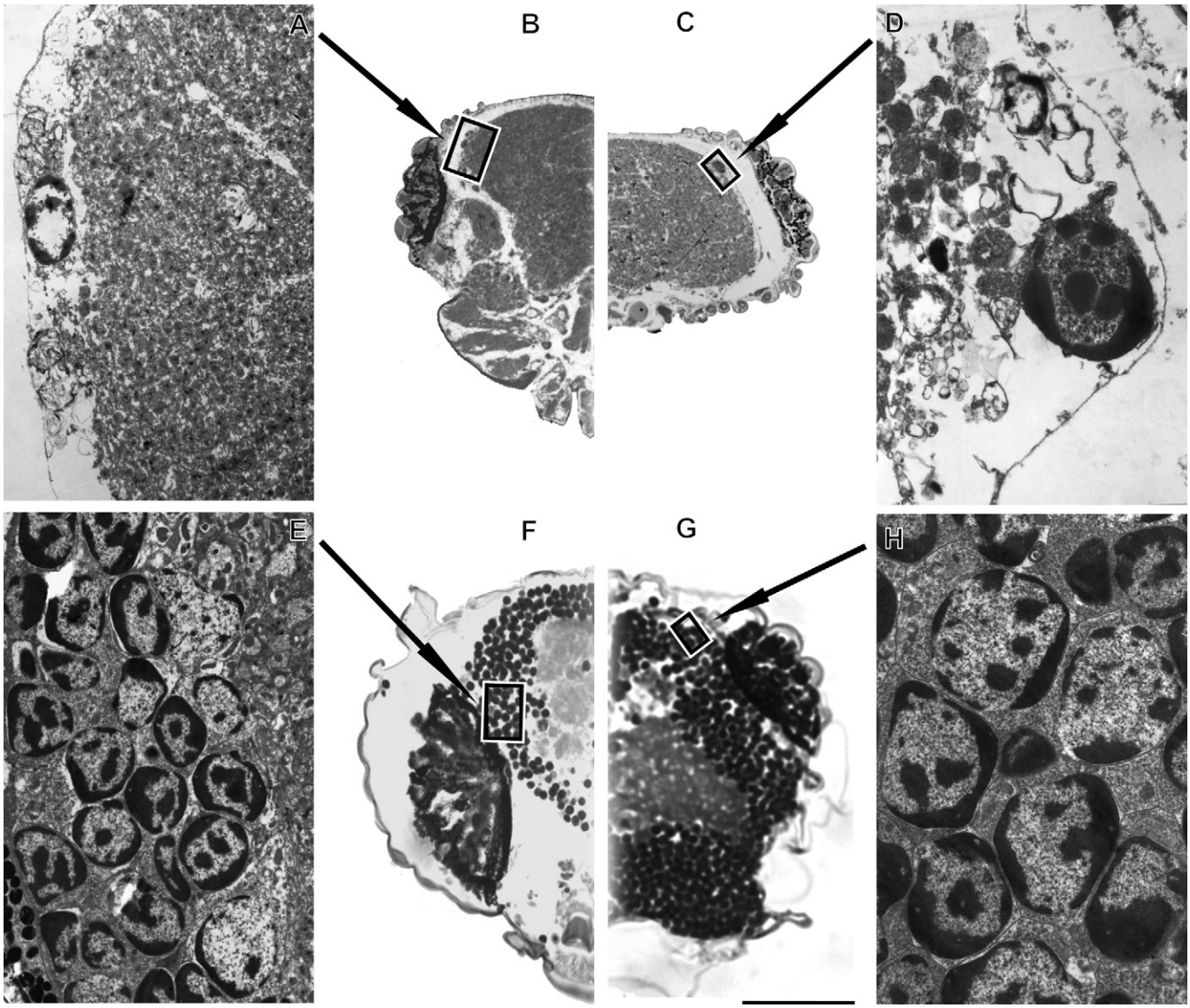

Fig. 3. Supraesophageal ganglion in *M. mymaripenne*. (A) Cortex layer of the ganglion of the adult with sporadic nuclei, cross section, TEM. (B, C) Section of the head of the adult, cross (B) and longitudinal (C), TEM. (D) Cortex layer of the ganglion of the adult with sporadic nuclei, longitudinal section, TEM. (E) Cortex layer of the ganglion of the pupa with multiple nuclei, cross section, TEM. (F, G) Section of the head of the pupa, cross (F) and longitudinal (G), Histology. (H) Cortex layer of the ganglion of the pupa with multiple nuclei, longitudinal section, TEM. Scale bar for A and E is 4 μm; for B, C, F, G is 25 μm; for D and H is 2 μm.

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convex occipital area, while in later pupae and adults the occipital area shrinks by forming numerous folds of the cuticle (Fig. 4). Each of these folds is a helically twisted cuticular area. Such transformations of the head capsule are likely a relatively widespread phenomenon among the smaller parasitic hymenopterans. Many Trichogrammatidae and Mymaridae have similar folds on the vertex and occipital parts of their head, the function of which are not known.

4. Discussion

Estimates of the anatomy of the nervous system and the size of neurons, limited by the volume of chromatin and axon diameter, are conservative, making the volume of the nervous system one of the principal factors limiting the decrease of body size (Kaas, 2000; Polilov, 2008; Polilov and Beutel, 2010). This problem is especially

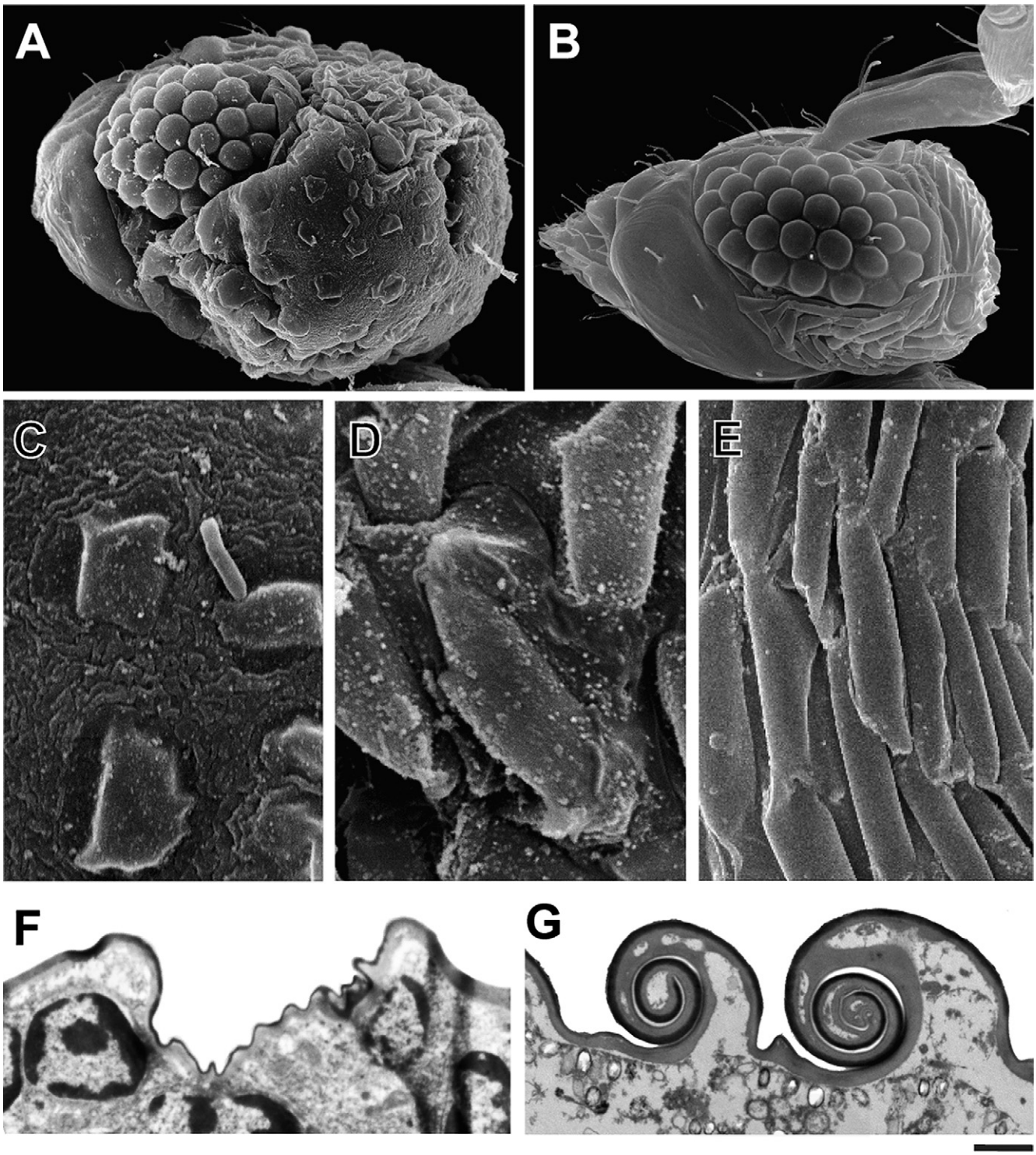

Fig. 4. Head capsule transformation after brain formation in *M. mymaripenne*. (A) Head capsule of pupa, SEM. (B) Head capsule of adult, SEM. (C–E) Fragment of cuticle in occipital area pupa (C), late pupa (D) and adult (E), SEM. (F, G) Section of cuticular folds in occipital area in pupa (F) and adult (G), TEM. Scale bar for A, B is 10 μm, for C–E is 2 μm, for F and G is 0.5 μm.

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acute for the brain, the largest structure in the CNS. The problem of an excessively large brain is solved in most small insects by a partial or complete shift of its position into thoracic, and in some larvae even abdominal, segments (Beutel et al., 2005; Polilov and Beutel, 2009). In hymenopterans, however, this is not possible because the head is very movable and the connection to the thorax is constricted. Thus the smallest hymenopterans have to resort to "extreme" changes in the structure of the nervous system, such as those described here.

The nervous system of *M. mymaripenne* is unique not only in the lysis of over 95% nuclei, but also in containing the smallest number of neurons in all known insects and all animals capable of flight. The approximate number of cells in the central nervous system of other members of this species is about 7400, 4600 of which are located in the brain. In larger insects, the number of neurons is higher by several orders of magnitude. For example, the brain contains about 340,000 neurons in *Musca* and about 850,000 neurons in *Apis* workers (Strausfeld, 1976). Clearly, smaller insects are characterized by a smaller number of neurons, but even the closely related *T. evanescens* has 37,000 neurons in the supraesophageal ganglion.

In spite of the small number of neurons, adult *M. mymaripenne* retain many important behaviors, including flight, feeding, and the ability to search for hosts for oviposition. This capacity of anucleate neurons provides crucial insight into the study of neuron regeneration. The functionality of axons isolated from the nucleus has been shown *in vitro* for many animal species (Bittner, 1988, 1991; Sotnikov et al., 2010), but intact anucleate neurons *in vivo* have never been described. The average lifespan of adult *M. mymaripenne* is 5 days at a temperature 25 °C and 8.8 at 15 °C (Bernardo and Viggiani, 2002), which is comparable to that of many larger members of Chalcidoidea. Thus, an almost anucleate nervous system of the adult is capable of sustaining all its functions over the relatively long adult life of the insect.

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