Cricket tympanal organ revisited: morphology, development, and possible functions of the adult-specific chitin core beneath the anterior tympanal membrane

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The number of text pages:

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Abstract

Vertebrates and insects are phylogenetically separated by millions of years but have commonly developed tympanal membranes for efficiently converting airborne sound to mechanical oscillation in hearing. The tympanal organ of the field cricket *Gryllus bimaculatus*, spanning 200 µm, is one of the smallest auditory organs among animals. It indirectly links to two tympana in the prothoracic tibia via tracheal vesicles. The anterior tympanal membrane is smaller and thicker than the posterior tympanal membrane, and it is thought to have minor function as a sound receiver. Using differential labeling of sensory neurons/surrounding structures and three-dimensional reconstructions, we revealed that a shell-shaped chitin mass and associated tissues are hidden behind the anterior tympanal membrane. The mass, termed the epithelial core, is progressively enlarged by discharge of cylindrical chitin from epithelial cells that start to aggregate immediately after the final molt, and it reaches a plateau in size after six days. The core, bridging between the anterior tracheal vesicle and the fluid-filled chamber containing sensory neurons, is supported by a taut membrane, suggesting the possibility that anterior displacements of the anterior tracheal vesicle are converted into fluid motion via a lever action of the core. The epithelial core did not exist in tympanal organ homologs of meso- and metathoracic legs or of nymphal legs. Taken together, the findings suggest that the epithelial core, a potential functional homolog to mammalian ossicles, underlies fine sound frequency discrimination required for adult-specific sound communications.

Key words: epithelial cells; tracheal vesicles; insect; cricket; chitin; tonotopic organization

Abbreviations: AtC: attachment cell; AN: auditory nerve; BM: basement membrane; ATM: anterior tympanal membrane; ATV: anterior tracheal vesicle; CD: ciliary dilation; CM: covering membrane; DD: dendritic dilation; DG: distal group neurons; EC: epithelial cell; ECL: epithelial cell layer; LAC: large accessory cell; La: labyrinth; Oli: olivarius organ; PG: proximal group neurons; PTM: posterior tympanal membrane; PTV:

posterior tracheal vesicle; SC: scolopale cell; ScC: scolopale cap; ScR: scolopale rod; SGO: subgenual organ.

Introduction

In order to detect airborne sound in terrestrial environments for acoustical communication, both vertebrates and insects have evolved tympanated hearing organs. Since both animal groups are phylogenetically distant, this is thought to be a typical example of convergent evolution (Hoy and Robert 1996). The emergence of tympanal membranes in prothoracic tibiae of ensiferan insects dates back at least to the Eocene era, and the ensiferan hearing organ is therefore one of the “oldest” tympanate ears that have adapted to the terrestrial environment for more than 50 million years (Rust et al., 1999; Plotnick and Smith 2012; Strauß and Lakes-Harlan 2014). They are backed by an air-filled space or cavity and innervated by a chordotonal sensory organ that consists of bipolar sensory neurons (Field and Matheson 1999; Yack 2004).

Several lines of study have shown similarity between vertebrate and insect hearing organs not only in external morphology but also at the molecular level. As an example, mechanosensory channels and functional molecules expressed in auditory sensory cells are largely shared between the fruit fly chordotonal organ in the antennal base (i.e., Johnston’s organ) and mouse auditory hair cells (Senthilan et al. 2012). For example, 217 of 274 Johnston’s-organ-associated genes have mouse or human orthologs, attributable to common ancestors (Senthilan et al. 2012). Active amplification of sound found in vertebrate hair cells has also been found in tympanal organs of hemimetabolous insects (Kössel et al. 2008; Mhatre and Robert 2013) and in Johnston’s organs of holometabolous insects (e.g., Robert and Göpfert 2002).

The similarity at the sensory neuronal level, however, might not be surprising given that the developmental and evolutionary origins of tympanal organs are derived from proprioceptive chordotonal organs that retain features of ancestral mechansensory organs (Boyan, 1993; Hoy and Robert 1996; Niwa et al. 2004). Insects have independently evolved tympanate hearing organs at least seventeen times, resulting in a large diversity in their bodily locations, sizes, and numbers of sensory neurons (Yager 1999). Thus, one might expect that “true” convergent evolution rather lies in sound-transmitting apparata adopted between animals, such as humans and insects.

A recent study revealed that sound transmission in humans and that in tree crickets share remarkable similarities in three fundamental processes (Montealegre-Z et al. 2012): 1) sound detection by a tympanal membrane(s), 2) converting a large displacement of vibration to a small but powerful pressure by a lever system, which functionally parallels to ossicles in mammals. 3) converting a mechanical pressure to fluid motion that underlies the frequency discrimination of sensory neurons.

The tympanal organ of field crickets *Gryllus* and *Teleogryllus* (fami ly: Gryllidae; subfamily: Gryllinae) has been one of the most intensively studied hearing organs in insects (Huber et al. 1989). It is the smallest in animals known so far, spanning only 200 μm despite its coverage of broad-band sound frequencies from 4 kHz to greater than 42 kHz (Esch et al. 1980; Hutchings and Lewis 1981). Seventy sensory cells are tonotopically arranged to detect higher frequency sound by more distally located neurons (Oldfield et al. 1986). Field crickets have two tympanal membranes of unequal sizes, in contrast to those in tettigoniids and tree crickets that possess similar sizes of tympana in the proximal tibia (Ball and Field 1981; Mhatre et al. 2009; Montealegre-Z et al. 2012).

The functions of the two tympanal membranes in field crickets have been evaluated by their ablation, immobilization, and measurements of mechanical properties. The posterior tympanal membrane (PTM, see Fig. 1a,b) is thin and six-times larger than the anterior tympanal membrane (ATM, see Fig. 1c; Young a nd Ball 1974a). The PTM vibrates with about ten-times larger amplitudes than those of the ATM (Johnstone et al. 1970). Another study showed that the ATM vibrates with 20 dB larger amplitude than neighboring cuticle and also about 20 dB below the vibration amplitude of the PTM (Larsen, 1987). Covering the PTM with Vaseline (Paton et al. 1977) or with wax (Huber et al. 1984) and detaching the PTM from the leg cuticle (Nocke 1972) all significantly decreased the sensitivity of auditory sensory neurons by 15-30 dB. In accordance with these findings, covering the external surface of the ipsilateral PTM with water abolished neural response in an auditory interneuron (Kleindienst et al., 1983). From these findings, the PTM is thought to be the primary sound receiver, and the motion of the PTM is crucial for hearing (Kleindienst et al. 1983).

In contrast, the function of the ATM has remained controversial. For example, occluding the ATM with petrolatum did not affect the sensitivity

of auditory interneurons (Hill 1974), but removal of the ATM resulted in cessation of activity of sensory neurons that tune to sound above 12 kHz (Nocke 1972). Huber et al. (1984) demonstrated that crickets with their ATM and PTM blocked have auditory thresholds nearly 30 dB higher than those in crickets in which only the PTM is blocked, suggesting a complementary function of the ATM.

In principle, the field cricket tympanal organ acts as a four-input system in which sound reaches the ipsilateral and contralateral internal walls of acoustic spiracles and the ipsilateral and contralateral surfaces of the posterior tympanal membranes, although the effect of sound input from the contralateral regions is small (Larsen and Michelsen 1978; Michelsen et al. 1994), supporting its function as a pressure-difference receiver. However, how sound input to the PTM is eventually converted to mechanical deformation of sensory cilia is still enigmatic. So far, cellular and subcellular features of tympanal organs in field crickets have been studied mainly in sectioned materials (Michel 1974; Young and Ball 1974a).

The aim of this study was to provide a comprehensive view of sound transmission pathways including the functionally enigmatic ATM in this smallest insect ear. It is scarcely understood how tympanal organ morphologies affect the frequency tuning properties of sensory neurons. To tackle this problem, we have developed low-invasive dissection techniques that leave the connections from tympanal membranes to sensory neurons intact (Nishino and Sakai 1997) but permit observations using a confocal laser scanning microscope. The three-dimensional reconstructions of tympanal organs allowed us to scrutinize fine anatomical links among the two tympanal membranes, tracheal vesicles and the main body of the tympanal organ. The results provide new insights into the function and development of the ATM and related structures that should be useful for future modelizing of the tympanal organ in biomechanics.

Materials and Methods

Animals

Male and female nymphal and adult field crickets, *Gryllus bimaculatus* DeGeer, at 0–15 days after the imaginal molt in a laboratory colony were used. They were maintained in a constant dark and light cycle (L:D = 12:12) at a temperature of 26˚C. Over 220 individuals were used for the experiments, though the data presented here were derived from a much smaller numbers of animals. Three male and three female adult field crickets, *Teleogryllus occipitalis*, collected at Amami Islands and kept in the laboratory for 12 days were also used for inspecting the tympanal organ structures from a comparative viewpoint.

Retrograde labeling of sensory neurons

The prothoracic tympanal organ and its homologs in the meso- and metathoracic tibiae, termed tracheal organs (Young and Ball 1974b; Eibl 1978), were investigated. Animals were briefly anesthetized by carbon dioxide. A leg of interest was amputated at the trochanter and the distal section was embedded in a plastic wax with the anterior side up on a beeswax plate. The main leg nerve 5 (N5B2) and the anterior sensory nerve running along the main trachea (N5B1) were exposed by removing the anterior cuticle and were immersed in a drop of cricket saline (Nishino and Sakai 1997). The two nerves were cut with microscissors (Vananas Scissors, 501778, WPI, FL, USA) and the distal cut-ends were picked up by an electrolitically tapered tungsten rod (ID: 200 μm) with the tip slightly bent and were placed in the tip of a tapered glass electrode filled with microruby (dextran, tetramethylrhodamine and biotin, 3000 MW, ThermoFisher Scientific). Care was taken not to stretch the nerve when handling. The preparation was left in a humidified chamber at 5 °C for 24 hours. The anterior region of the epicuticle except for the ATM in the proximal tibia was carefully stripped off with fine microscissors to leave the translucent hypodermis intact (Fig. S1). The removal of the epicuticle allowed 1) the interior structure to be left nearly intact, 2) promotion of permeabilization of the fixative and fluorescent dye for counterstaining in the proximal tibia

rich with air-filled space (see below) and 3) scanning through the whole tympanal organ along the Z-axis without interference under a confocal microscope. The leg was pre-fixed in 4 % neutral paraformaldehyde solution for 15 min at room temperature.

Counterstaining and immunolabeling

Counterstaining with a fluorescent dye was used to visualize the anatomical link between the tympanal organ sensory neurons and surrounding structures such as tympanal membranes and tracheal vesicles. For differential staining, the specimens in which sensory nerves had been labeled with microruby were immersed in 0.5% Lucifer Yellow CH (Sigma Aldrich) in cricket saline (Nishino and Sakai 1997) for 40 min after pre-fixation (Nishino et al. 2016). To visualize F-actin filaments, the fixed specimens were transferred to 2.5% phalloidin solution (Acti-stain™ 488 Fluorescent Phalloidin, Cytoskeleton, Inc) for 1 h at room temperature before dehydration (Wolfrum 1990). After careful removal of the anterior tympanal membrane, chitinase from Streptomyces (C6137, Sigma) was applied to the epithelial core and surrounding tissues at 37˚C for 3 h in phosphate buffered saline set at pH 6.0 to confirm whether these are made up from chitin. Chitinase is an extracellular enzyme complex that degrades chitin and has a molecular mass of approximately 30 kDa. Chitin is degraded to N-acetyl-D-glucosamine in two enzymatic reactions. Firstly, chitobiose units are removed from chitin by chitodextrinase-chitinase. The second reaction involves N-acetyl-glucosaminidase-chitobiase, which cleaves the disaccharide to its monomer subunits (that comprise N-acetyl-D-glucosamine). All of the specimens processed by these treatments were thoroughly rinsed with cricket saline and post-fixed in 4% neutral paraformaldehyde solution for 1-2 h at 5 °C, dehydrated in an ethanol series, and cleared in methyl salicylate.

Microscopic observation of fixed specimens and data processing

The cleared specimens stained with fluorescent dyes were observed under a confocal laser scanning microscope (LSM 5 Pascal, Zeiss) and a light microscope (Imager Z1, Zeiss). Specimens differentially labeled with

Lucifer yellow and microruby were visualized with an argon laser with a 505–530-nm bandpass filter and a helium-neon laser with a longpass filter (>560 nm), respectively. Optical sections were made at a resolution of 1024 x 1024 with 0.6-2.0-µm intervals. Plan-Apochromat x10 0.8-NA and x20 0.8-NA (dry objectives) were used for observation of the entire tympanal organ complex, and Plan-Neofluar x40 1.3-NA (oil immersion objective) was used for observation of components of the tympanal organ. Observations were firstly made on wholemount preparations. Then transverse sections of the tibia at 100~200 µm in thicknesses were made manually by using a twin blade razor (0.1 mm in thickness, Feather, Japan) after the specimens had been rehydrated in 70% ethanol.

The optical sections were converted to TIFF-formatted files using the software LSM Image Browser (Carl Zeiss, Jena, Germany). TIFF images were processed with image processing software (Amira ver. 3.1, Visage imaging, Berlin, Germany). The sensory neurons and surrounding structures were manually outlined in each optical section for subsequent three-dimensional representations using a volume rendering function. Three-dimensional surface models of internal structures of the legs were obtained by surface rendering.

All photographic images were processed using Adobe PhotoShop Elements 6 (San Jose, CA). In figures, two to twenty consecutive optical sections at different focal planes were overlaid as aligned stacks and flattened to one plane.

Terminology

We referred to Young and Ball (1974a) for the naming and terminology of different kinds of cells associated with the tympanal organ, to Ribeiro and Brehelin (2006) for naming and classification of hemocytes, to Nishino and Sakai (1997) for terminology of nerves, to Young and Ball (1974b) and Eibl (1978) for the tracheal organ (counterparts of the tympanal organ in the meso- and metathoracic tibiae), and to Young and Ball (1974a) for orientations of the tympanal organ and tracheal organ (see Fig. 1a). The numbers of sample sizes are shown in parentheses in Results, wherever needed.

Results

Three-dimensional structure of the tympanal organ

The tympanal organ in field crickets is an assembly of nine distinct apparata as shown in the two-dimensional optical stacks (Figs. 1d-h, 2a-f, ESM1) and three-dimensional reconstructions (Figs. 1i, j, 2 g, h) of the proximal tibia of *G. bimaculatus* (Fig. 1a-c). These apparata (Fig. 1i, j) include 1) the posterior tympanal membrane (PTM, yellow), 2) the posterior tracheal vesicle (PTV, dark blue), 3) the anterior tracheal vesicle (ATV, light blue), 4) a tent-like cellular mass (red), 5) a thin covering membrane interconnecting the lateral side of the mass and tracheal vesicles (CM, Fig. 1f), 6) sensory neurons (magenta) on the ATV, innervated by the auditory nerve (AN, Fig. 1d) diverged from N5B1, 7) the anterior tympanal membrane (ATM, light yellow), 8) the core structure hidden beneath the ATM. It attaches postero-distally to the covering membrane (green; Fig. 2 f, g, l), and 9) lipid-synthesizing organs that clog hemolymph channels proximo-distally to the cellular mass, termed the olivarius organs in primitive tettigoniids, wetas (Oli, Fig. 1d, ESM1; Lomas et al. 2013).

The PTM, which is the primary sound input site, has a hinged structure in its central to distal region (Fig. 1g; see also ESM7), reminiscent of tettigoniid tympanal membranes (Bangert et al. 1998). The inflated PTV, tightly apposed to the PTM, is located parallel to the smaller ATV (Fig. 1j, ESM2). The PTV and ATV are interconnected via two apertures (Fig. 1h, j, ESM2; Eibl 1978).

The tent-like mass is composed of two types of cells: 1) attachment cells (AtCs), each receiving insertion of a sensory neuronal cilia, and 2) large accessory cells (LACs) that primarily contribute to building up the mass (Fig. 2a; Young and Ball 1974a). The AtCs and LACs are readily identifiable by the shape of their nuclei, the former being more ellipsoidal (Figs. 2a, 3a; ESM1). All of the sensory neurons extend dendrites into single attachment cells at one-to-one correspondence via scolopale caps (SCs, Figs. 2a, c, e, 3a, ESM1). There was no indication that dendrites of proximal neurons of the distal group extended directly to modified hypodermal cells that attach to the hypodermis, as suggested in *T. commodus* (Young and Ball 1974a). The cellular mass is attached to the

dorsal cuticular ceiling via a bundle of approximately 30 ligaments originating from hypodermal cells (inset, Fig. 1e). The attachment site is thinner than surrounding cuticles (arrow, Fig. 1e). The bundle of ligaments radiate ventrally and cover the whole cellular mass (Fig. 1e; ESM1).

The covering membrane (CM) is thin, approximately 0.5 μm in thicknesses (Fig. 1f). The membrane is secreted by unidentified cells with diameters of 5-7 μm that line the inside of the membrane (inset, Fig. 1f). The membrane descends along the lateral edge of the cellular mass to attach to the anterior region of the dorsal membrane of the PTV and the anterior edge of the dorsal membrane of the ATV (ESM1). The plasma serum is often coagulated inside the covering membrane through a fixation process (e.g., see ESM6), suggesting that the sensory dendrites are immersed in a lipidic fluid (Huber et al. 1989). In all of the fixed specimens with interior structures left intact, no hemocytes were observed around sensory dendrites (Figs. 1f; 2a; ESM1), though aggregation of a few hemocytes was often observed in the proximo-posterior region of the fluid chamber (Fig. 2f; ESM1).

The sensory neurons are classified into a proximal group (PG, Fig. 2a-d, g) and a distal group (DG, Fig. 2b, e, f, h) based on whether dendrites are attached to the cellular mass externally or immersed in fluid. Based on this criterion, only proximal group A defined by Young and Ball (1974a) was categorized into the proximal group and other neurons were categorized into the distal group. Neurons in the proximal group, a group of neurons that is a possible functional homolog of the intermediate organ in wetas (Ball and Field 1981; Nishino and Field 2003; Strauß et al. 2017), extend dendrites along the antero-dorsal to postero-ventral region of the cellular mass (Fig. 2d). Proximal group neurons attach to the AtCs at their ciliated region of dendrites (Fig. 3b), where mechanosensory channels are distributed (review: Li et al. 2018).

Neurons in the distal group are arranged proximo-distally on the dorsal membrane of the ATV (Fig. 2b, e, f), of which thicknesses are approximately 1-2 μm (Fig. 2e, f; ESM1). As shown in the dorsal view, somata and dendrites of the distal group of neurons are arranged diagonally at about 45˚ against the longitudinal axis of the tibia, in which the more distal cells occupy the more anterior region (Fig. 2c, h). This arrangement allows maximal use of the space in the limited dimension of the tympanal

organ. Dendrites of the distal group of neurons bridge between the dorsal membrane of the ATV and the bottom of the cellular mass (Fig. 2e-g, j). The dendrites of the distal 20 neurons of the distal group extend to AtCs elongated from the bottom of the cellular mass (Fig. 2a; ESM1). Their dendritic arrangements and orientations are topographically complex. The array of sensory dendrites winds like an S shape (broken lines in Fig. 2i, k), in which dendrites of more proximal neurons extend into the attachment cells at smaller angles due to the narrower spaces between the ATV and the cellular mass in the proximal direction (Fig. 2e, f; ESM1). This is in reverse sequence for the tettigoniid *crista acustica*, in which distances between the attachment cells and the dorsal membrane gradually become larger in the proximal direction (Hummel et al. 2017). Dendrites located in the middle region of the distal group neurons tended to be denser than those in the proximal and distal ends (Fig. 2c, i).

Being consistent with our previous findings (Nishino et al. 2016), phalloidin stainings showed that scolopale rods of sensory neurons are rich in actin filaments (Fig. 3b-d), and peripheral regions of large accessory cells showed weak immunoreactivity to actin filaments (Fig. 3a). Subcellular structures, such as the scolopale cap (ScC), ciliary dilation (CD), and dendritic dilation (DD), typical of scolopidia participating in auditory/vibratory detection (Field and Matheson 1998; Yack 2004), were detectable in dendritic cilia (Fig. 3b, d).

Structures of meso- and metathoracic tracheal organs

Counterparts of the prothoracic tympanal organ, termed tracheal organs in the meso and matathoracic tibiae (Young and Ball 1974b; Eibl et al. 1978), resemble the premature tympanal organ in nymphs (Ball and Young 1974). Tracheal organs are not equipped with tympanal membranes or core structures (Fig. 4). As shown by Young and Ball (1974b) and by Eibl (1978), only sensory neurons corresponding to proximal group A are present (Fig. 4c, d). The non-inflated tracheae corresponding to the ATV and PTV run in parallel but are not interconnected by two apertures (Fig. 4a, b). Olivarius organs adjacent to the cellular mass are present in meso- and metathoracic tracheal organs (Fig. 4a, b). Closer observation revealed that unlike the tympanal organs in phalate adults (Fig. 4e-g) and 6th instar

nymphs (Fig. 4h), which possess a well-developed covering membrane, there were no covering membranes in tracheal organs (Fig. 4c, d). Instead, the cellular mass was suspended to the anterior cuticular wall via fine fibers (Fig. 4c; ESM3), resembling those seen in the *crista acustica* homologs in the atympanate ground weta *Hemiandrus pallitarsis* (Strauß et al. 2017). Since hemolymph passage is permitted beneath the cellular mass, a number of hemocytes were detectable beneath the cellular mass of meso and matathoracic tracheal organs (Fig. 4c, d; ESM3).

Epithelial core and associated tissues in the mature adult

The epithelial core, previously termed the “tracheal body” (Friedman 1972a, b; Huber et al. 1989), exists in *G. bimaculatus* and *T. occipitalis* but only in mature adults (Figs. 2g and 5). Mature cores are shell-shaped and are approximately 140 µm x 90 µm square and 60 µm in thickness in *G. bimaculatus* (Fig. 5a-e) and approximately 110 µm x 60 µm square and 25 µm in thickness in *T. occipitalis* (Fig. 5f-h). The designation of tracheal body is, however, inappropriate because the core is neither part of the trachea nor a product secreted by tracheoblast cells (Wigglesworth 1954; Affolter and Causinius 2008). Instead, the core is built up by external scaffolding of epithelial cells (also referred to as epidermal cells, Wigglesworth 1959) to the dorso-anterior edge of the ATV. Since the core is adhered to a mechanically unstable position and protrudes anteriorly like a lever (Fig. 6i-n), the core needs to be supported by surrounding tissues for keeping its original position. As shown in three-dimensional reconstructions of the core region (Fig. 5) and transverse sections (Fig. 6), the following six features were detectable beneath the ATM.

1) The basement membrane (BM) that extends from the dorsal rim of the ATM approaches the core and attaches nearly perpendicularly to the fringe of the antero-dorsal surface of the core (Fig. 5a, b, d; ESMs4, 5), the site being slightly more ventral than that of the covering membrane on the opposite side (ESMs4, 5). The BM is composed of a layer of single cells and is 4-5 µm in thickness (ESM5). The width of the membrane becomes smaller in the distal direction, as shown in the dorsal view (Fig. 5b). The antero-proximal region of the BM is firmly attached to the ATM (ESM5).

2) The attachment site of the BM to the core is wedged by epithelial cell layers (ECLs, Fig. 6l-n) that run in parallel along the anteriormost surface of the core (ESMs4, 5). The ECLs are embedded in a sheet of whitish connective tissues (see Fig. 9a). The thicknesses of ECLs are approximately 7 µm near the core and 20 µm in the ATM rim area (ESM5). The complex of BM/ECLs is taut and not flaccid, literally like a tympanal membrane.
3) The space between the complex of BM/ECLs and the ATM is a chamber immersed in hemolymph that contains a few hemocytes and epithelial cells (see Fig. 8e, f; ESM5).
4) There are labyrinth-like frameworks that loosely support the proximo-ventral side of the core (ESMs4, 5). A bundle of fibers mechanically bridges the ECLs and the anterior wall of the ATV/ventral region of the core (Figs. 5c, 6b-f). The cavities made by labyrinth are immersed in hemolymph that contains numerous plasmatocytes (ESMs4, 5).
5) The posterior surface of the core is attached to the fluid-filled chamber at its distal two thirds (Figs. 2l, 6j, k, m, n; ESMs6, 7). Contact of the core with the fluid is not direct but via a thin covering membrane. The covering membrane is often detached from the core in the proximal half region in *T. occipitalis* (e.g., Fig. 6i; ESM6), while it attaches to the central region of the proximal edge of the core in *G. bimaculatus* (Fig. 8f; ESM5).
6) As in the PTV supported ventrally by the cuticular ledge (Fig. 6a-h), the ATV on which the main body of the tympanal organ lies is supported ventrally by a thick cuticular ledge that protrudes posteriorly from the rim of the ATM (Fig. 6c-f; ESM7; Michel 1974; Eibl 1978).

The epithelial core is hard, but surrounding tissues are elastic

The epithelial core itself is a shell-shaped, hard, and translucent mass (Fig. 7a). The core was degraded by placing a small amount of chitinase powder onto it and subsequent incubation at 37 ˚C for 3 h (Fig. 7b), suggesting that the core is made up by N-acetylglucosamine, which is the primary monomeric unit of the polymer chitin. The ligaments connecting the

cellular mass to the hypodermis were also degraded by chitinase, resulting in collapse of the cellular mass onto the ATV (Fig. 7d). The covering membrane and tent-like cellular mass maintained their structures after application of the chitinase (Fig. 7c), suggesting that they have a composition other than chitin.

The elasticity of the basement membrane and that of epithelial cell layers were assessed in a fresh prothoracic leg with the epicuticle of the ATM and the surrounding epicuticle carefully removed (ESM8). This treatment leaves the structures underlying the ATM intact. Pressure applied to the exocuticle of the distal femur caused a volumetric increase in the ATV and PTV because tracheal systems are closed in the tibia and, in turn, air in the extracellular space surrounding the main body of the tympanal organ pushes the basement membrane away in an outward (anterior) direction (n=8, ESM8), suggesting that the melanized basement membrane is elastic but sufficiently hard to push away the epithelial cell layers ventrally. The movement is larger in the proximal region of the basement membrane than in the distal region (ESM8). Such movements were never observed in intact specimens, suggesting that the movement of the basement membrane is normally hindered by its suspension to the ATM in the proximal region.

Progressive development of the epithelial core and associated tissues

In the nymphal and phalate adult of *G. bimaculatus*, the covering membrane is attached to the interior wall of the anterior cuticle (Fig. 4e, f). Therefore, the space between the cellular mass and the anterior cuticle is narrow (ca. 60 μm). Moreover, the ATV and the PTV are not directly attached but separated by tracheoblast cells (arrow, Fig. 4f; Wigglesworth 1954).

The core and labyrinth are concurrently formed between the ATM and ATV. Immediately after the final molt, epithelial cells with diameters of 4-5 μm aggregate beneath the ATM, forming cell layers (Figs. 8a, 9a). The layers are especially thick in the ventro-distal region beneath the ATM (ESMs4, 5). The epithelial cells produce thin filopodium-like processes laterally to connect themselves to each other (Fig. 8b). Some of the epithelial cells invaginate by elongation of thick filopodia (Fig. 8c),

allowing their migration toward the future core region. The epithelial cells remaining just beneath the ATM secrete chitin outward (anteriorly), which contributes to thickening of the ATM from 8.13 ± 0.58 μm immediately after the final molt (n = 3, Fig. 8c) to 19.78 ± 0.79 μm in a mature adult (measured by Schneider et al., 2017).

A sheet of single cells with diameters of 6-8 μm, probably plasmatocytes, is static from the final molt and becomes the basement membrane (Fig. 8d, e, Wigglesworth 1973). The intercellular space appears to be filled with connective tissues discharged by plasmatocyes (Wigglesworth 1956). The basement membrane is gradually melanized over a period of one week (see Fig. 9a, b). The small space between the basement membrane and the ATM is maintained throughout the adult stage (Fig. 8e, f, ESM5; Friedman1972a).

In contrast, plasmatocytes that aggregated ventral to the future core region are active. The plasmatocytes phagocytize filopodium processes of epithelial cells and shape them into frameworks in the labyrinth, except for the anterior-most epithelial cell layers (Fig. 8g; ESMs4, 5). Debris of the filopodia of epithelial cells is continually removed away by phagocytosis by plasmatocytes that stay beneath the ATM through adult life (ESMs4, 5).

The core augmentation occurs progressively. At the end of day 1, invaginated epithelial cells are scaffolded onto the ATV and start to secrete chitin inward (Fig. 9c). This is the beginning of core formation. On day 2, individual epithelial cells start to discharge chitin material (Fig. 8h-j). Three-dimensional reconstructions of tractable chitin materials in the core (Fig. 8i, j) showed their cylindrical appearances, corresponding to those found by Friedman (1972b) in *G. assimilis*. The chitin cylinders collectively form a small fan-shaped core (Figs. 9d). From day 3, concomitant with inward recruitment of more epithelial cells, the core grows accretionally (Fig. 9e-h) and enlarges toward dorso-anteriorly until the size reaches a plateau on day 6 (Fig. 9i). The enlarged core gradually separates the ATV from the ATM, making the cellular mass 120 μm away from the ATM in the mature adult (Fig. 6l, ESM5).

The epithelial cell nuclei lining the posterior surface of the core are orderly arranged like a fan (Fig. 8k, l). The early epithelial cells scaffolded onto the ATV undergo apoptosis and become themselves part of the core. Scaffolding of epithelial cells, chitin secretion, and attachment of new

epithelial cells to the cell bodies of old cells should be precisely regulated spatio-temporally so that accretionary growth of the shell-shaped mass occurs (Fig. 8m).

Formation of the epithelial core is independent of innervation of peripheral nerves

The formation of the epithelial core is a robust process that is unaffected by denervation after the imaginal molt. For example, decerebration (including the corpus allatum) and denervation of N5B1 (innervating the tympanal organ) and N5B2 (innervating part of the SGO) in the proximal tibia immediately after the imaginal molt resulted in normal core formation and associated structures (n = 5). Cutting all of the peripheral nerves unilaterally close to the prothoracic ganglion immediately after the final molt also resulted in normal formation of the core and associated structures. Thus, the core augmentation proceeds without control of sensory neurons or efferent neurons in the adult stage. Keeping animals in constant dark from the final instar to adult stage also resulted in normal formation of the core (n = 7), suggesting that core formation is not affected by light impinging through the translucent ATM or by light-entrained circadian rhythms.

Discussion

Overview

Studies on tympanal organs in field crickets have a long history dating back to Schwabe's anatomical study (1906) and Autrum's physiological study (1941). In contrast to detailed descriptions about frequency tuning of auditory sensory neurons (Oldfield, 1986; Pollack and Imaizumi 1999; Imaizumi and Pollack 1999, 2005), the key structure transmitting the mechanical energy to the sensory neurons has remained enigmatic. The epithelial core, first discovered by Michel (1974), was only briefly referred to in a monograph (Huber et al., 1989). The tissue embedding the core, termed the suspensorium (Schwabe 1906; Michel 1974), has been thought to be a mechanical damper (Larsen and Michelsen 1978).

Our study revealed, for the first time, that the ATM has a bilayering structure: a thick outer membrane and an underlying thin membrane complex composed of a basement membrane and epithelial cell layers. The epithelial core, bridging between the ATV and the fluid-filled sensory chamber, is supported by a taut membrane complex. There are no energy-absorbing structures around the core, suggesting more active functions other than a passive mechanical damper. On the other hand, the outer membrane of the ATM is 20 μm in thickness and has no mechanical links with the internal structure. Thus it is reasonable that immobilizing the outer membrane had no effect on the physiological properties of sensory neurons in *T. commodus* (Hill 1974) or on acoustic orientation in *T. oceanicus* (Bailey and Thomson 1977). All sound transmission apparata including the trachea, the epithelial core, and tympanal membranes that are made up by chitin or materials polymerized with chitin will be important for impedance matching on sound transmission between their junctions.

The meso- and metathoracic counterparts of the prothoracic tympanal organ, tracheal organs, retain evolutionary conserved features (Yager 1999; Strauß and Lakes-Harlan 2014). These organs lack an epithelial core, covering membrane, and distal group neurons (that tune to high-frequency sounds), suggesting an intimate functional link of the core with high-frequency sound detection in the fluid-filled environment.

The core development is precisely regulated but independent of peripheral neuronal control. The hearing system in the cricket, therefore, offers not only a model for understanding a miniature ear achieving a broad audible range but also a model for dynamic cell migration based on self-assembly (Fig. 8m), nowadays extensively studied in developmental biology (e.g., Ma et al. 2017; Matsubaysahi et al. 2017).

Our histological procedures permitted observation in almost intact specimens. Generally, tissues connected to the exoskeleton cause inadvertent deformation of the structure through formaldehyde fixation. Moreover, embedding agents such as epons hardly penetrate into the cuticle, resulting in deformation or even loss of internal tissues in sectioned materials. Young and Ball (1974a) failed to find the epithelial core in *T. commodus*, presumably due to the loss of the core during sectioning. Removal of the exocuticle and two-step fixation allowed wholemount confocal observations of semi-intact specimens at sufficiently high

resolutions (Fig. 3), which are beyond the resolutions of micro CT (> 3 μm).

Possible sound transmission pathways

Several lines of studies in field crickets have shown that the PTM is crucial for hearing (Johnstone et al. 1970; Paton et al. 1977; Huber et al. 1984) and that it acts as a pressure-difference receiver that compares external sound impinging onto the PTM with internal sound impinging to the tracheal opening in the prothorax and transmitting to the internal vesicle (Larsen and Michelsen 1978; Larsen 1987; Michelsen et al. 1994). The PTM is broadly tuned with an optimum well above the calling song frequency (Lankheet et al. 2017; Schneider et al. 2017). Its tuning is not sharp enough to account for the tuning of individual sensory neurons, which is generally more selective (Oldfield et al. 1986; Hutching and Lewis 1981; Imaizumi and Pollack 1999). These findings suggested the existence of a second filter somewhere in the tibia (Larsen et al. 1989). In this context, our findings provide insights into how sound energy is mapped in a frequency-dependent manner for individual sensory neurons.

Structures surrounding the core

In general, sensory neurons beneath the cellular mass, i.e., the distal group of neurons, are mechanically isolated from structures other than tracheal vesicles. The regions proximal and distal to the tympanal organ are protected by olivarius organs, the thick ATM, and cuticular ledges. These structures will prevent the disturbance of hemolymph passage and the disturbance of muscular movements to the tympanal organ. Moreover, the ATV is largely surrounded by incompressible fluid, that is, the fluid-filled sensory chamber, the ventral hemolymph channel, and labyrinth, except for the antero-distal edge to which the core is attached. We assume that the labyrinth has two functions: 1) frameworks, made from filopodia of epithelial cells, mechanically support the ventral side of the core loosely. 2) the cavities in the labyrinth encloses hemolymph, creating a static environment immersed in fluid.

In such a protected environment, the sound impinging to the PTM could be transmitted effectively between solid media, from the large PTV

to the small ATV, and to the core, all of which are appositioned in parallel (Fig. 10c, d). Two sound transmission pathways are conceivable from the anatomy of these pathways. First, the thin region of the PTV can resonate with the PTM vibration and transmit sound energy to the ATV via its junction. Since the thin dorsal membrane of the PTV is attached directly to the fluid-filled chamber, longitudinal waves of sound components may enter the fluid and affect the sensitivity of sensory neurons (Greenfield, 2002), although there are no specialized structures that effectively compress the fluid laterally in the junction between the PTV and ATV (Fig. 10c, d). Second, compressional waves of sounds can lead to instantaneous displacements of the ATV. Airflows through the two apertures potentially result in a volumetric increase of the ATV and push the interior wall outward. Although it is not known whether either of these or both contribute to the stimulation of sensory neurons, instantaneous displacement of the ATV would be more effective for core displacement.

Core - is it designed to convert mechanical displacement to fluid motion?
Neurons of the distal group are immersed in hemocyte-free fluid sealed by the covering membrane. The dendrites of these neurons are arranged with dendrites of distal neurons oriented progressively more perpendicularly against the dorsal membrane and occupying the wider fluid space between the dorsal membrane and the cellular mass. Neurophysiological studies have shown that sensory neurons are tonotopically organized so that more distal neurons in the distal group tune to sound with higher frequencies (Oldfield et al. 1986). The fluid-filled chamber is attached to the core in the distal region via the thin covering membrane (Fig. 10a, b). From these findings, it is tempting to speculate that the core is ideally situated for converting its mechanical displacement to fluid motion, which is the direct source of mechanical deformation of the cilia of the neurons in the distal group.

The core has topographically different connectivities with surrounding tissues (Fig. 10a-d). The proximal region of the core, inflated in *G. bimaculatus*, is supported anteriorly by complex of the elastic basement membrane/epithelial cell layers, but only loosely supported ventrally by labyrinth-like frameworks (Fig. 10b, c). The distal region of the core is firmly supported by thick epithelial cell layers (dark blue, Fig. 10d). Along

the dorso-ventral axis, the ventral side of the core is adhered to the ATV in a large area (yellow line, Fig. 10a, b), but the attachment site of the core to the covering membrane is limited to the distal two thirds (Fig. 10b).

Given these features, we propose that a class 1 lever action operates on the core (Fig. 10e, f). The membrane complex is taut like a tympanal membrane and is backed by a small chamber immersed in incompressible hemolymph (Fig. 10e). The membrane complex attaches to the core at anteriormost areas so that the core in the stationary state is immobilized by a delicate balance between the fluid-filled chamber, the labyrinth, and the membrane complex (Fig. 10e). Therefore, when the core attachment to the ATV is displaced outward, i.e. anteriorly (thin arrow, Fig. 10e), a small but powerful inward pressure will be generated at the attachment site to the fluid chamber (thick arrow, Fig. 10e). Moreover, the proximal inflated region of the core is attached to the anterior wall of the ATV from the lateral direction (Fig. 10c), and the distal thin region of the core is attached to the corner between the dorsal membrane and the anterior wall of the ATV (Fig. 10d). Due to this geometry, the outward pressure to the anterior wall of the ATV can displace the proximal region of the core anteriorly but displace the distal region of the core more dorso-posteriorly. This complementary lever operating proximo-distally might help to generate a diagonal fluid motion from the disto-anterior direction to the proximo-posterior direction and subserve frequency discrimination of sensory neurons that are also arranged diagonally along the proximo-distal axis (Fig. 10g). The presumed core action in field crickets is, in principle, parallel to the action of the tympanal plate of the tettigoniid *Copiphora gorgonensis*, which is attached to the fluid chamber containing sensory neurons and that vibrates 180˚ out of phase against the thin tympanal membranes (Monteraegre-Z and Robert 2015).

Further physiological investigations are needed to determine the extent to which the fluid motion driven by the core contributes to generation of traveling wave and frequency discrimination. The only available data are results of a study showing that there was no electrical response of the auditory nerve to tones above 12 kHz after ablating the ATM in *G. campestris* (Nocke 1972). Nevertheless, this is in good accordance with the finding that the most distal 20 neurons that tune to sounds higher than 15 kHz (Huber et al. 1989) are ideally situated for receiving a fluid current.

Since high-frequency sounds tend to attenuate during transmission in the medium, distal neurons might need assistance from fluid motion for sharp frequency tuning. Systematic disruption of the core integrity is needed for elucidating concrete core functions in the future.

Functional aspects of two neural groups

The cricket tympanal organ is composed of two anatomically defined neuronal groups; the proximal group and the distal group. While the somata of the proximal group are firmly suspended to the anterior cuticular wall, their dendritic tips are inserted to the cellular mass beyond the apertured area. This aperture possibly limits propagation of vibration transmitted through the longitudinal ATV and permits detection of the displacement of the mass against the soma attachment site. Adequate stimulus of the proximal group, therefore, would be displacements of the mass along the anterior-posterior axis (Fig. 10a), a scheme similar to the subgenual organ in which sensory dendrites attach to the ellipsoidal cellular mass (Nishino and Field 2003; Strauß et al. 2012). However, the primary sources of vibration that evoke displacement of the mass are still enigmatic. The ligaments connecting the cellular mass to the thinned cuticle are one possible route for transmission of cuticular vibration to proximal group neurons.

The stimulus situation in distal group neurons is also complex even though their dendrites are immersed in fluid. The two apertures appear to limit transmission of sound energy to the ATV region between them (Fig. 10a). However, tensile stress could be loaded on the cilia not only directly by the fluid motion but also indirectly by displacement of the cellular mass and/or the dorsal membrane of tracheal vesicles (Bangert et al. 1998).

Physiological data imply the presence of such complex interplay between the cellular mass and fluid-filled chamber. A striking characteristic of cricket sensory neurons equivalent to the distal group is the occurrence of additional sensitivity peaks at frequencies other than characteristic frequencies (Esch et al. 1980; Hutching and Lewis 1981; Imaizumi and Pollack 1999). Additional sensitivity peaks of auditory receptors often emerge when sounds with higher intensities are applied (Imaizunmi and Pollack 1999). Therefore, such complex responses of single sensory

neurons may be attributable to the proximo-distal gradation of connectivity strength with surrounding tissues; i.e., distal neurons may be more sensitive to the core motion than to the cellular mass motion and in reverse sequence for proximal neurons.

Our assumption does not exclude the importance of frequency filtering based on intrinsic properties of scolopidial sensilla (Oldfield et al. 1986). Since morphological specializations of dendrites, scolopale caps, and attachment cells are evident among individual neurons of the distal group (Young and Ball 1974a), the stiffness gradient between dendritic cilia and attachment cells might confer additional frequency filtering to sensory neurons, as suggested in *crista acustica* of Tettigoniidae (Hummel et al. 2017). In fact, tuning properties of sensory neurons are invariable from the last instar that has no epithelial cores, although the auditory thresholds of sensory neurons are 30-45 dB higher in last instars than in adults (Ball and Hill 1978; Staudacher 2009).

The developmental data suggested the importance of the distal group of neurons in adult life. In contrast to the proximal group of neurons, the number of which is invariable from the fourth instar, the number of neurons in the distal group increases progressively from the sixth instar to the adult stage (Ball and Young 1974). Sound-transmitting apparata linked to the distal group of neurons, such as tracheal vesicles and the epithelial core, progressively maturate after the imaginal molt. Maturation of the core, which takes six days after the imaginal molt, is important for transmitting sufficiently powerful pressure into the fluid. The thresholds of acoustic orientation behaviors to the calling song with carrier frequency 5 kHz in female crickets gradually decrease by 20-30 dB in about 11 days after the imaginal molt (Sergejeva and Popov 1994). The improvement in sensitivity after the imaginal molt may be, at least partly, attributable to the formation of the epithelial core.

Evolutionary perspective

The tympanal organ of the field cricket (Gryllinae) differs greatly from the *crista acustc*a of tettigoniidae in that it has unequal-sized tympanal membranes and no clear differences in sensory neuronal sizes. Despite its translucent appearance like a specialized membrane, the ATM is

acoustically less sensitive than the PTM (Larsen, 1987; Huber et al. 1989; Schneider et al., 2017). Given that tympanal membranes in Gryllidae are morphologically diverse (Huber et al., 1989; Schneider et al. 2017), one might speculate that this system reflects habitats and behavioral ecology unique to field crickets.

In a “tree-thinking” perspective, the subfamily Eneopterinae species are phylogenetically close to Gryllinae but have adapted to tropical rainforests (ter Hofstede et al. 2015; Chintauan-Marqier et al. 2016). The tribe Lebinthini *L. bitaeniatus* has an ATM covered with a cuticular fold, in addition to the PTM (Schneider et al. 2017). The ATM is smaller than the PTM and the structure of the tympanal organ resembles that of field crickets, retaining conserved features between Gryllinae and Eneopterinae. However, the ATM of *L. bitaeniatus* is thinner than the PTV, which results in sensitivity reversal of the PTM and ATM between *G. bimaculatus* and *L. bitaeniatus* (Schneider et al. 2017). On the other hand, another tribe, Eurepini, that has adapted to ground and shrubs in Australia (Grandcolas et al. 2010), has a PTM but no ATM (Schneider et al. 2017). These results indicate the possibility that their habitats, in addition to their unique acoustic communications (ter Hofstede et al. 2015), affect ear morphology in these cricket species.

In field crickets, 1) adaptation to ground habitats and 2) ultrasound detection for avoiding bats could be reflected in morphologies of the tympanal organ. Field crickets, in general, have habits that dig burrows in the ground for shelter (Michelsen 1998; Gawarek et al. 2014). Eibl (1978) found long sensory hairs close to the PTM that presumably detect burrowing movements in field crickets. Their habitats that have many hard obstacles such as stones and their burrowing habits raise the potential for damage to the thin part of the cuticle, i.e., tympanal membranes. Damage to the ATM leads to immediate dysfunctioning of the tympanal organ because it is in close proximity to the fluid-filled sensory neurons sealed by the thin covering membrane. This situation differs from that of the PTM, which is indirectly attached to the tympanal organ via the PTV. Since sound impinges not only to the PTV but also to the tracheal opening in the prothorax (Larsen and Michelsen, 1978; Poulet and Hedwig, 2001), sound transmission through the PTV to the tympanal organ is still functional under condition of damage of the PTM (Kleindiest et al., 1983; Schmitz et

al., 1983). Therefore, strong selective pressure could be exerted on the ATM rather than on the PTV.

Sensitivity to ultrasound is thought to be acquired later than acquirement of an intraspecific communication system during an evolutionary process (Stumpner and von Helversen 2001; Strauß and Stumpner 2014). Since avoidance of ultrasound emitted by bats provides high survival value, modification of the pre-existing tympanal organ must have been needed for ultrasound detection at high sensitivity. Therefore, the thickening of the ATM and the core formation might be an evolutional solution for protecting the distal group of neurons from mechanical damage as well as enabling high-frequency sound detection. Thickening of the ATM and external core attachment are indeed achieved by a small modification of the pre-existing structure.

The core size and shape differ even between two field cricket species, *G. bimaculatus* and *T. occipitalis*, the former being larger and more inflated proximally. Comparative neuroanatomy between crickets equipped with a thick anterior membrane (Gryllinae), thin membrane (Lebinthini), and no membrane (Eurepini) will be useful for assessing functions of the cores and evolution of sound transmission pathways.

In conclusion, compared to tettigonnid ears in which bilateral compression of two equal-sized tympanal membranes produces fluid motion (Montealegre-Z and Robert 2015), the field cricket tympanal organ is a more perturbation-resistant ear, in which vibrations of the single tympanal membrane are likely converted to unilateral fluid motion via a lever action (Fig. 10g). In this context, the cricket ear systematically resembles mammalian ears that enclose ossicles deep in the middle ear (Fig. 10h). Measurements of mechanical properties of sound transmission pathways will be the priority toward application of this miniature ear for industrial use, as directed by a beautiful biomimetics example of the ear of the parasitoid fly *Orimia ochracea* to a hearing aid (Mason et al. 2001; Miles and Hoy 2006).

Acknowledgments

This work was supported by Innovative Materials Engineering Based on Biological Diversity in Grant-in-Aid for Scientific Research on Innovative Areas (project number: 80332477) to HN and TT. HN thanks Dr. Masnori

Ochiai (Hokkaido University) for expertized comments on identification of blood cells and epithelial cells and Dr. Larry Field (University of Canterbury) for valuable discussions from comparative viewpoints.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1. Prothoracic leg tympanal organ and surrounding structures in minimally dissected specimens. (a-c) Posterior tympanal membrane (PTM, b) and anterior tympanal membrane (ATM, c) in the prothoracic tibia (a). (d-j) Two-dimensional optical stacks (d-h) and three-dimensional reconstructions (i, j) of the tympanal organ in which sensory neurons are labeled by microruby and surrounding structures are counterstained with Lucifer Yellow. From posterior to anterior, the PTM, PTV (posterior tracheal vesicle), ATV (anterior tracheal vesicle), core, and ATM are appositioned in parallel (see also ESMs 1,2). The PTV and ATV are interconnected by two apertures (h, j, see also ESM2). Sensory neurons located between the cellular mass and the dorsal membrane of the ATV are immersed in lipidic fluid sealed by the covering membrane (CM). The CM is secreted by cells lining the membrane (inset in f). The cellular mass is suspended by a bundle of ligaments (inset in e) to the thinned cuticle (arrows in e). The distal two thirds of the PTM has a hinge-like structure, as shown in a transverse section (g). AN, auditory nerve; Oli, olivarius organ; SGO: subgenual organ. *Scale bars*: (a) 1 mm; (b-d) 500 µm; (e-g, inset in e) 50 µm; (inset in f) 10 µm; (h) 100 µm; (i, j) 200 µm.

Fig. 2 The main body of the tympanal organ. (a-h) The cellular mass is composed of small attachment cells (AtCs) and large accessory cells (LACs). Sensory neurons, viewed dorsally (b, c) and proximally (d-f), are grouped into the proximal group (PG) and distal group (DG). The numbers of cells in *g* and *h* are seven for the PG and 60 for the DG. The PG dendrites externally attach to the proximal edge of the cellular mass (d). The DG dendrites (b) and scolopale caps (ScCs, c) are orderly arranged proximo-distally. DG somata are firmly attached to the dorsal membrane of the ATV (e, f). Note that most distal neurons extend dendrites to attachment cells elongated from the cellular mass (a, f). (g, h) Three-dimensional reconstructions of sensory neurons, AtCs and LACs, showing that sensory dendrites bridging between the dorsal membrane of the ATV and the bottom of the cellular mass, gradually rise more perpendicularly to the membrane from proximal to distal. (i-k) Topographically complex arrangements of the dendritic cilia of the DG, viewed dorsally (i) and

distally (j, k). The orientations of dendrites and ScCs are schematized (i, k). (l) An optical section close to the covering membrane showing that the core attaches to the covering membrane at its' dorso-distal region. The outline of the core (broken line) is overlaid. SC: scolopale cell. Scale bars: (a-h, l) 50 µm; (i-k) 20 µm.

Fig. 3 Subcellular structures of sensory neurons revealed by phalloidin staining. (a) The LAC and AtC are readily identifiable by their spherical and ellipsoidal shapes of nuclei, respectively. (b-d) Different magnified images of the ciliated region of sensory dendrites showing that scolopale rods (ScR) rich in actin filaments highlighten an orderly arrangement of sensory dendrites and specialized structures such as the scolopale cap (ScC), ciliary dilation (CD), and dendritic dilation (DD). SC: scolopale cell. Scale bars: (a, c) 50 µm; (b, d) 10 µm.

Fig. 4 Meso and metathoracic tracheal organs (a-d) and tympanal organs in nymphs (e-h). (a-d) Meso and metathoracic tracheal organs have a cellular mass structurally resembling that in the prothoracic tympanal organ but lacking inflated tracheae interconnected by two apertures (a, b), distal group sensory neurons, covering membrane, and core (c, d). The cellular mass is suspended to the ATV via fine fibers (c, see also ESM3). Olivarius organs (Oli) are stuffed in the space distal and proximal to the tracheal organ and the subgenual organ (SGO). Note hemocytes aggregating beneath the cellular mass (c, d). (e-h) Tympanal organs in a phalate adult (e-g) and 6th instar (h) showing that the PTV and ATV are spaced by tracheoblast cells (arrow in f), but the covering membrane has already developed (g, h). The cellular mass is located closely to the anterior cuticle since the core has not developed yet (e, f). Scale bars: (a, b) 500 µm; (c, d, f-h) 50 µm; (e) 100 µm.

Fig. 5 Three-dimensional reconstructions (volume rendering) of the epithelial core of *G. bimaculatus* (a-e) and a smaller field cricket, *T. occipitalis* (f-h), in which the epicuticle of the ATM has been removed. The basement membrane (BM) that extends from the rim of the anterior tympanal membrane is attached to the antero-distal edge of the core (d, see also Fig. 9b). The width of the BM becomes smaller distally (b), resulting

in the distal region of the core being closer to the ATM (e). A smaller core is present in the field cricket *T. occipitalis* (f-h). ECL: epithelial cell layer; La: labyrinth. Scale bars = 50 μm.

Fig. 6 Transverse sections of the prothoracic tibia in *T. occipitalis* (a-k) and *G. bimaculatus* (l-n) showing varied association of the core with surrounding tissues proximo-distally. The apparata comprising the tympanal organ are overlaid on the optical sections (a-h). Numbers in a-h are distances from the proximal tip of the cellular mass. The attachment fashion between the PTV and ATV is varied from proximal to distal (a-h). The frameworks in the labyrinth (La) support the proximal region of the core from its ventral aspect (c, d). Contact of the core with the lipidic fluid is not direct but via the covering membrane (CM) (i, j), and only the distal region of the core attaches to the covering membrane (d-f, k, m, n). The basement membrane (BM) is attached posteriorly to the dorsal region of the core (j, k, l-n). Transverse sections distal to the cellular mass (i-n, see Fig. 2d-f for proximal regions) show that the CM keeps its tautness (l-n). Scale bars: (a-h) 100 μm; (i-n) 50 μm.

Fig. 7 Application of chitinase to the epithelial core. (a) The epithelial core was cleared in methyl salicylate and viewed under differential interference contrast. (b-d) Chitinase treatment resulted in breakdown of the core (b) and the ligaments (d) connecting the cellular mass to the dorsal cuticle, suggesting that these are made up by chitin. The cellular mass and covering membrane were not affected by application of chitinase (c). Scale bars: 100 μm.

Fig. 8 Cellular contribution to the core formation in *G bimaculatus*. (a-c) After the final molt, epithelial cells (ECs) with dimeters of 4-5 μm aggregate beneath the ATM (a) and interconnect laterally by thin filopodia (b). Some of epithelial cells (ECs) start to migrate posteriorly to attach to the ATV by producing thick filopodia posteriorly (c). (d, e) The basement membrane (BM) comprising a sheet of single cells with diameters of 7-8 μm (d) attaches to the periphery of the core anlage (e). (f) The proximal edge of the core attaches to the CM in its central region. The epicuticle of the ATM is removed. (g) The epithelial cell layers (ECLs) ventral to the

core region are shaped into the labyrinth (La) by phagocytosis of thin filopodia by plasmatocytes. (h-j) The ECs scaffolding on the ATV start to secrete chitin inside (h) at day 1 (see Fig. 9c). Three-dimensional reconstructions of tractable chitin cylinders show that epithelial cells secrete chitin inside and participate in enlargement of the chitin mass (i, j). (k, l) Mature core in which epithelial cells are radiated like a fan. New epithelial cells attach to the cell bodies of old cells and secrete chitin (arrowheads in l). (m) Working hypotheses of core development. The core is formed by repetition of 1) epithelial cell scaffolding to the ATV, 2) secretion of chitin, and 3) new cells scaffolding to old cells. Note that epithelial cells located laterally tend to secrete thicker but shorter chitin compared to the central region, collectively contributing to accretionary growth of the fan-shaped core. Scale bars: (a, d-g, k, l) 50 μm; (b, c) 10 μm; (h-j) 20 μm.

Fig. 9 Epithelial core development. (a, b) Intact ATM viewed under an objective microscope in adults at different ages, showing that epithelial cells embedded in whitish connective tissues aggregate beneath the ATM at day 1 (a) and the melanized basement membrane (BM) attaches to the edge of the core at day 6, demarcating the core (outlined by a broken line in b). (c-h) Progressive enlargement of the core from day 1 to day 6. (i) Volumetric measurement of the core during development showing that the core size reaches a plateau at day 6, although some inter-individual differences are evident (n= 5 for each day). Scale bars: (a, b) 100 μm; (c-h) 50 μm.

Fig. 10 Sound transmission pathways in the tympanal organ of field crickets. (a) Schematized representation of sound transmission apparata viewed anteriorly. (b) Schematic representation of elaborated structures underlying the ATM. The three attachment sites to the core and the BM to ATM attachment site are shown in red. (c, d) Different connectivities of the proximal region of the core (e) and the distal region of the core (d) with the ATV and the membrane complex. (e, f) Possible lever action to the core. Outward inflation of the ATV results in inward displacement of the dorsal core due to the support of the membrane complex functioning as a fulcrum. This is class 1 lever action (f). (g, h) The core action (g) is presumably

functionally parallel to that of mammalian ossicles (h) in that they both convert large mechanical displacements to small but powerful pressures in the fluid. See text for abbreviations. Scale bars: (a) 500 μm; (b-e) 100 μm.

Legends of Supplementary Materials

SM1 Procedures for low-invasive observation of the interior structure of the tympanal organ in *G. bimaculatus*.

ESM1 Adult tympanal organ in the left prothoracic tibia of *G. bimaculatus*, viewed anteriorly, showing sensory neurons (magenta) and associated structures (green) including the epithelial core.

ESM2 Tympanal organ of the left prothoracic tibia of *G. bimaculatus*, viewed dorsally, showing parallel appositions between the PTM, PTV, ATV, core and ATM. Note two apertures interconnecting the PTV and ATV. See text for abbreviations.

ESM3 Tracheal organ in the left mesothoracic tibia of *G. bimaculatus*, viewed anteriorly, showing no epithelial core, no covering membrane and no distal group neurons. The cellular mass is suspended to the anterior cuticle wall and the dorsal membrane of the ATV via thin fibers. Note hemocytes aggregating beneath the cellular mass. SGO: subgenual organ.

ESM4 ATM of the left prothoracic tibia of *G. bimaculatus*, viewed anteriorly, showing elaborate structures associated with the core. The epicuticle of the ATM was carefully removed in this specimen. See text for abbreviations.

ESM5 Tympanal organ in the left prothoracic tibia of *G. bimaculatus*, viewed proximally, showing different connectivities of the core with surrounding structures. The epicuticle of the ATM was removed in this specimen. Note that a small air-filled space between the ATV and the complex of the basement membrane/epithelial cell layers becomes larger distally. The labyrinth-like frameworks, originating from filopodia of epithelial cells, bridge between the epithelial cell layers and ATV and core.

The central region of the proximal tip of the core attaches to the covering membrane. The epicuticle of the ATM and that of the surrounding exoskeleton were removed in this specimen. See text for abbreviations.

ESM6 Tympanal organ in the left prothoracic tibia of *T. occipitalis,* viewed proximally, showing the covering membrane attached to the distal region of the core. Note the lipidic fluid coagulated inside the covering membrane. See text for abbreviations.

ESM7 Low-magnified image of the tympanal organ in the left prothoracic tibia of *T. occipitalis*, viewed proximally, showing different attachments between the PTV and ATV proximo-distally. Note thick ledges protrude from the ATM to support the ATV ventrally. See text for abbreviations.

ESM8 Movement of the basement membrane and the underlying core. The slow pressure-on to the exocuticle of the distal femur results in movement the basement membrane toward ventrally while the pressure-off results in return of the basement membrane to its original position. Note that the displacement of the distal core region is smaller than that of the proximal region.

Fig. 1

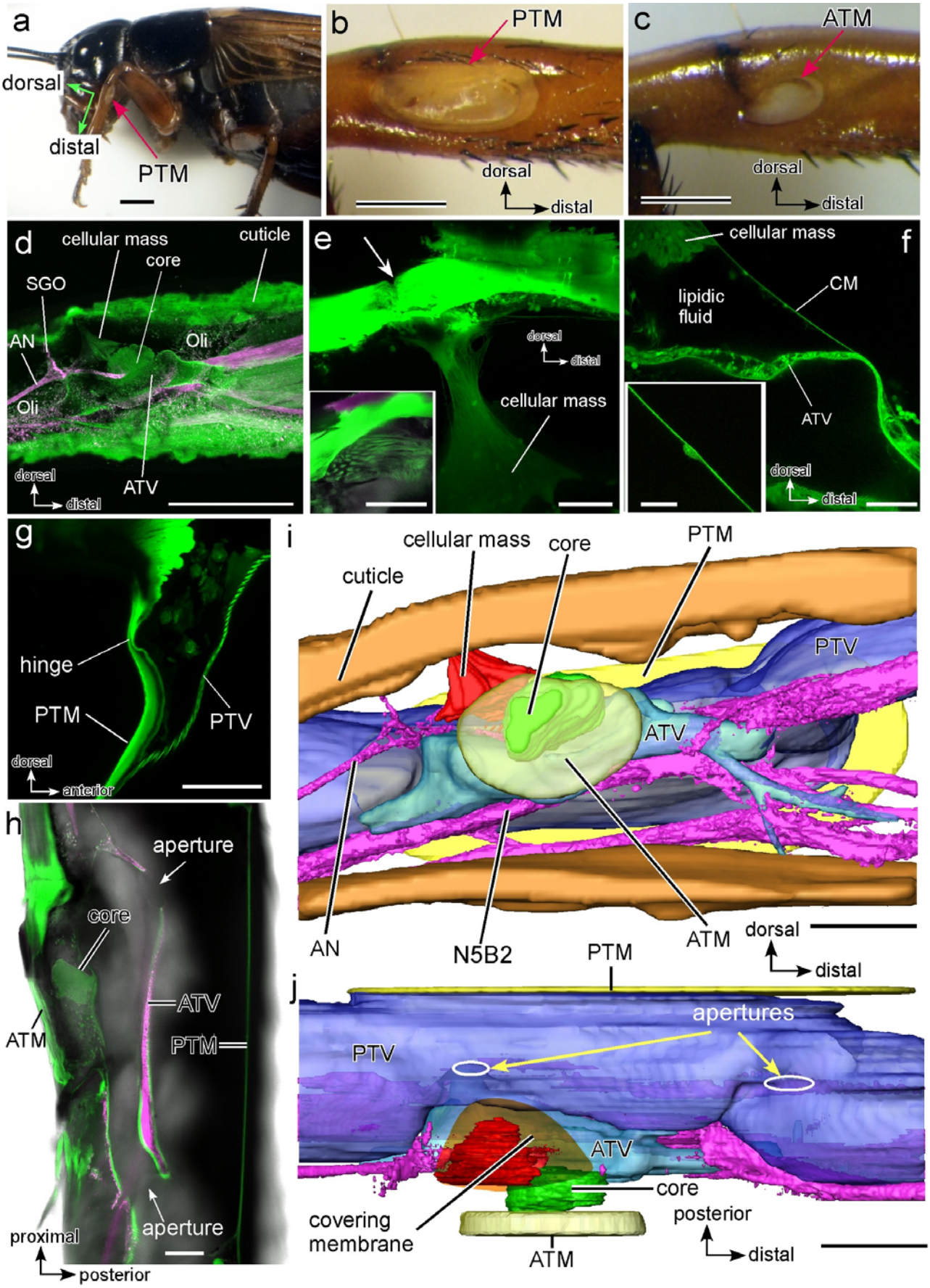
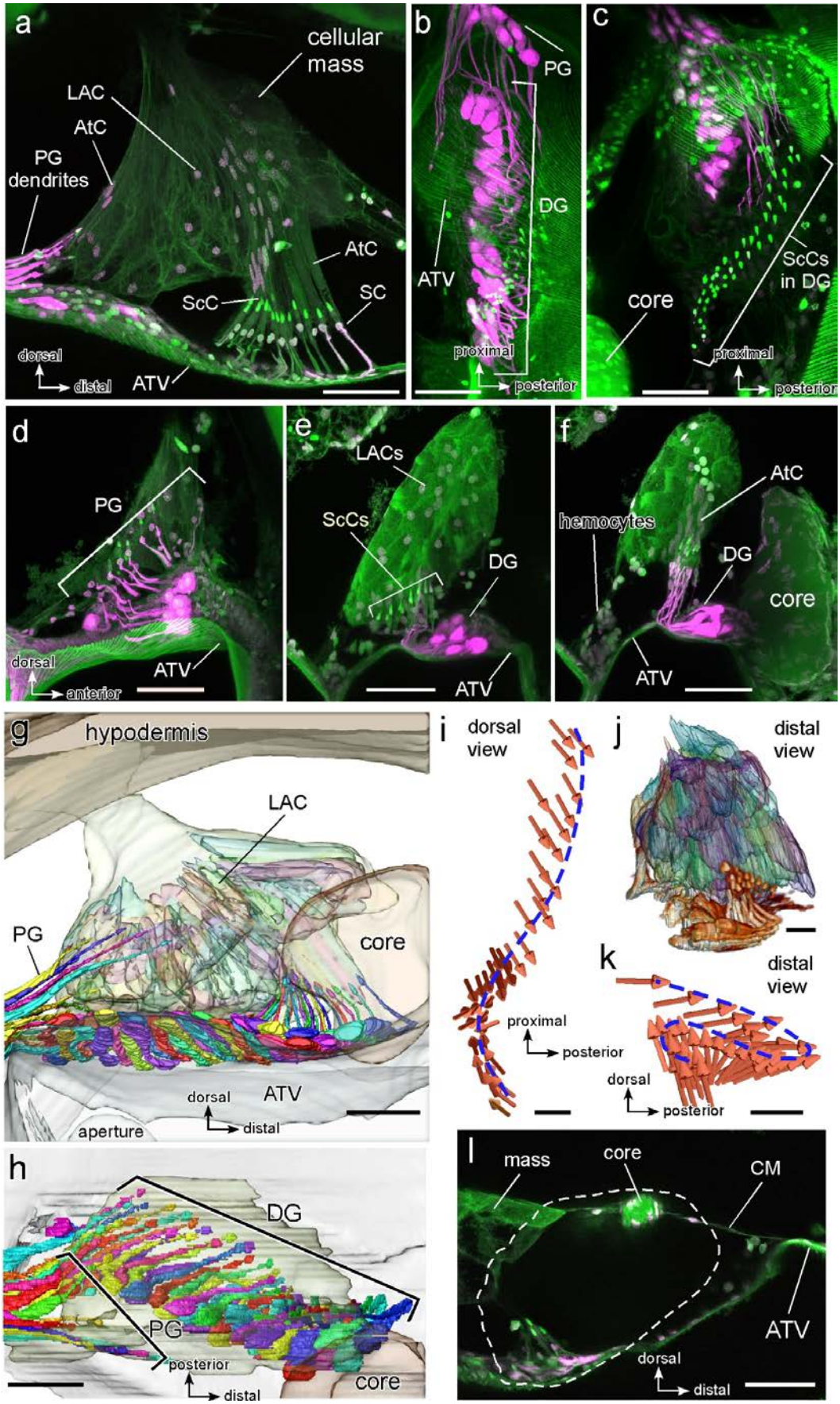

a
dorsal
distal
PTM
b
PTM
dorsal
distal
c
ATM
dorsal
distal
d
cellular mass
cuticle
core
SGO
AN
Oli
Oli
ATV
dorsal
distal
e
dorsal
distal
cellular mass
f
cellular mass
lipidic fluid
CM
ATV
dorsal
distal
g
hinge
PTM
PTV
dorsal
anterior
h
aperture
core
ATV
ATM
PTM
proximal
aperture
posterior
i
cellular mass
core
PTM
cuticle
PTV
ATV
AN
N5B2
ATM
dorsal
distal
j
PTM
apertures
PTV
ATV
core
covering membrane
ATM
posterior
distal

Fig. 2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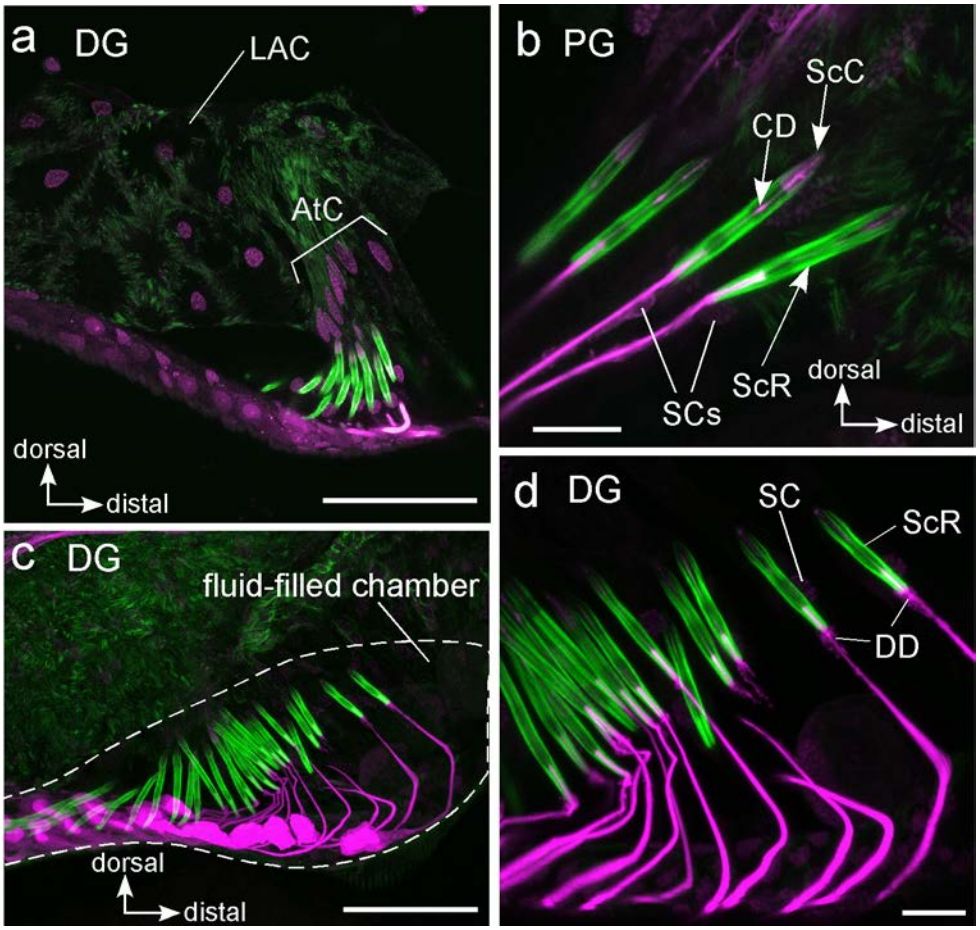
cellular mass
LAC
AtC
PG dendrites
AtC
ScC
SC
dorsal
distal
ATV
b
PG
DG
ATV
proximal
posterior
c
ScCs in DG
core
proximal
posterior
d
PG
dorsal
anterior
ATV
e
LACs
ScCs
DG
ATV
f
AtC
hemocytes
DG
core
ATV
g
hypodermis
LAC
PG
core
dorsal
ATV
distal
aperture
h
DG
PG
posterior
distal
core
i
dorsal view
proximal
posterior
j
distal view
k
distal view
dorsal
posterior
l
mass
core
CM
ATV
dorsal
distal

Fig. 3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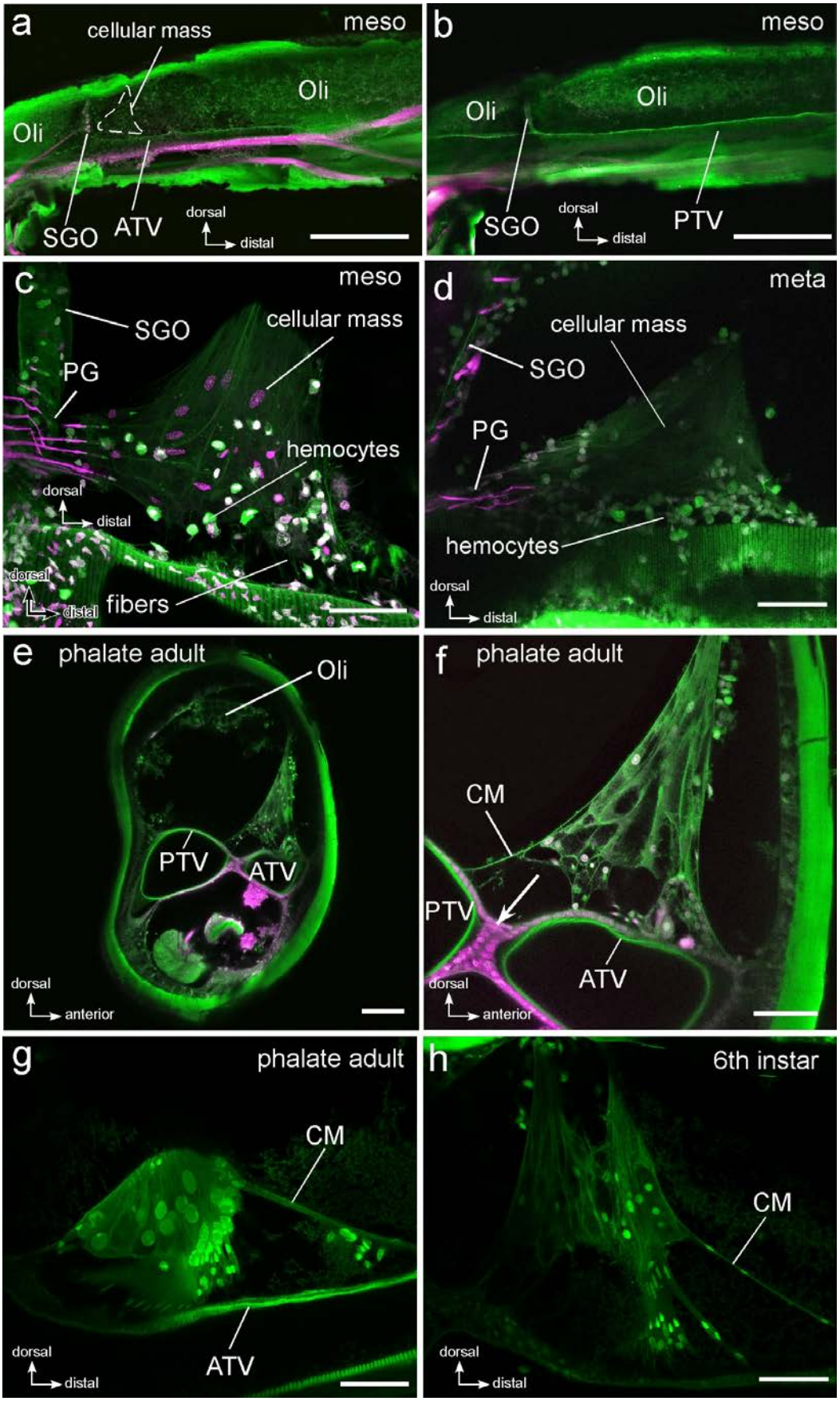
LAC
AtC
dorsal
distal
b PG
ScC
CD
SCs
ScR
dorsal
distal
c DG
fluid-filled chamber
dorsal
distal
d DG
SC
ScR
DD

Fig. 4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meso
cellular mass
Oli
Oli
SGO
ATV
dorsal
distal
b
meso
Oli
Oli
SGO
dorsal
distal
PTV
c
meso
SGO
cellular mass
PG
hemocytes
dorsal
distal
dorsal
distal
fibers
d
meta
cellular mass
SGO
PG
hemocytes
dorsal
distal
e
phalate adult
Oli
PTV
ATV
dorsal
anterior
f
phalate adult
CM
PTV
ATV
dorsal
anterior
g
phalate adult
CM
ATV
dorsal
distal
h
6th instar
CM
dorsal
distal

Fig. 5

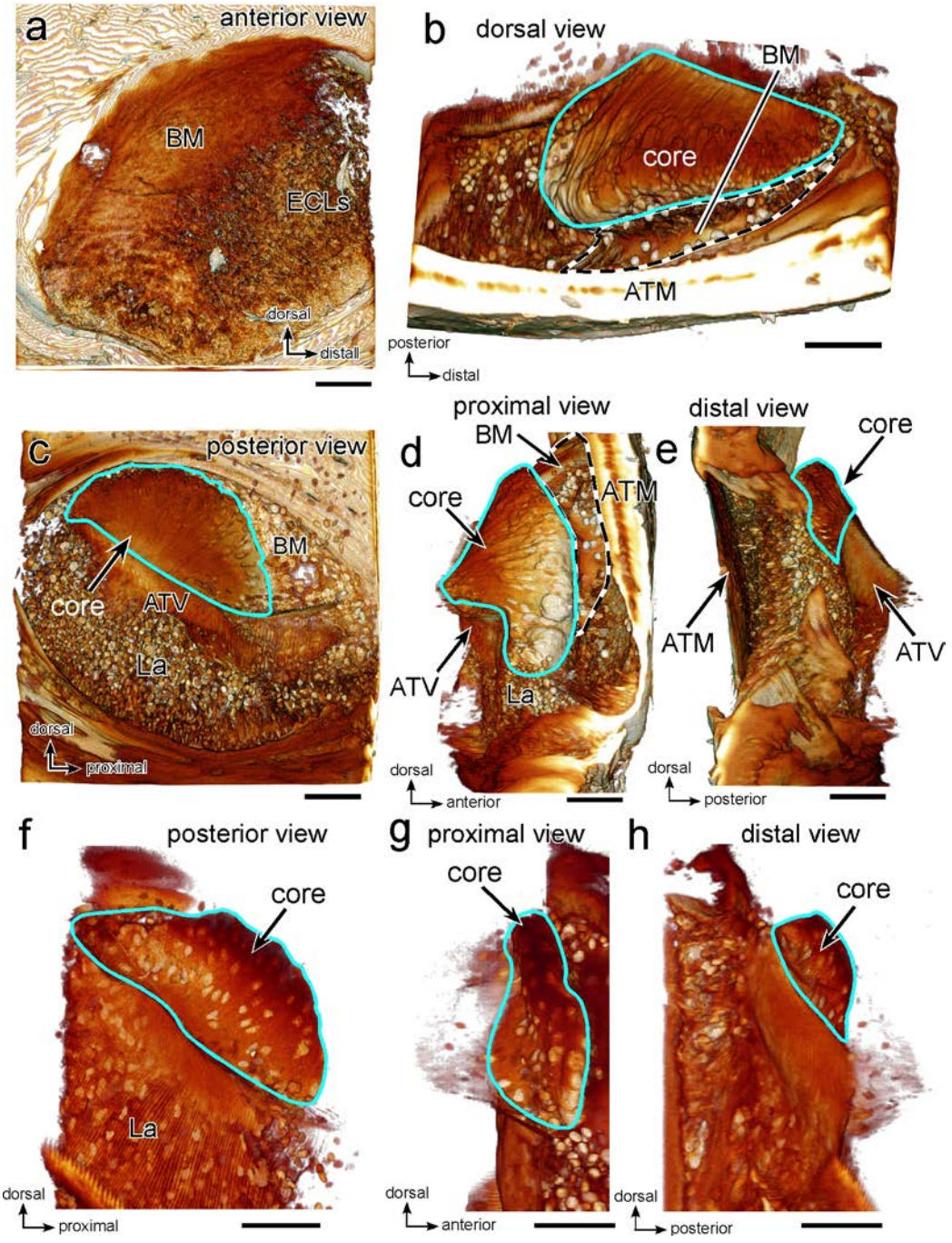

a
anterior view
BM
ECLs
dorsal
distal
b
dorsal view
BM
core
ATM
posterior
distal
c
posterior view
BM
core
ATV
La
dorsal
proximal
d
proximal view
BM
ATM
core
ATV
La
dorsal
anterior
e
distal view
core
ATM
ATV
dorsal
posterior
f
posterior view
core
La
dorsal
proximal
g
proximal view
core
dorsal
anterior
h
distal view
core
dorsal
posterior

Fig. 6

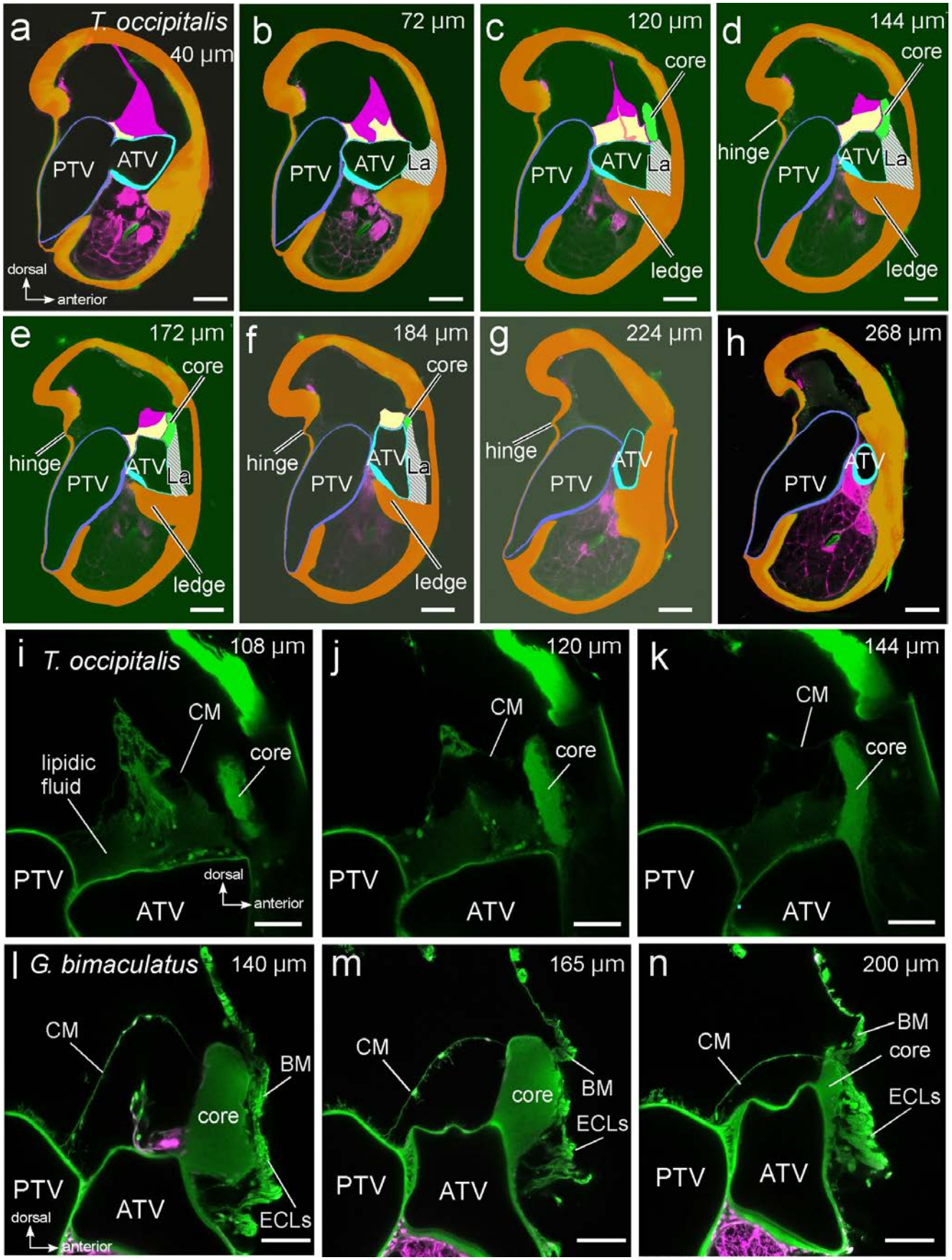

a
T. occipitalis
40 µm
PTV
ATV
dorsal
anterior
b
72 µm
PTV
ATV
La
c
120 µm
core
PTV
ATV
La
ledge
d
144 µm
core
hinge
PTV
ATV
La
ledge
e
172 µm
core
hinge
PTV
ATV
La
ledge
f
184 µm
core
hinge
PTV
ATV
La
ledge
g
224 µm
hinge
PTV
ATV
h
268 µm
PTV
ATV
i
T. occipitalis
108 µm
CM
core
lipidic fluid
PTV
dorsal
anterior
ATV
j
120 µm
CM
core
PTV
ATV
k
144 µm
CM
core
PTV
ATV
l
G. bimaculatus
140 µm
CM
BM
core
PTV
ATV
dorsal
anterior
ECLs
m
165 µm
CM
core
BM
ECLs
PTV
ATV
n
200 µm
BM
CM
core
ECLs
PTV
ATV

Fig. 7

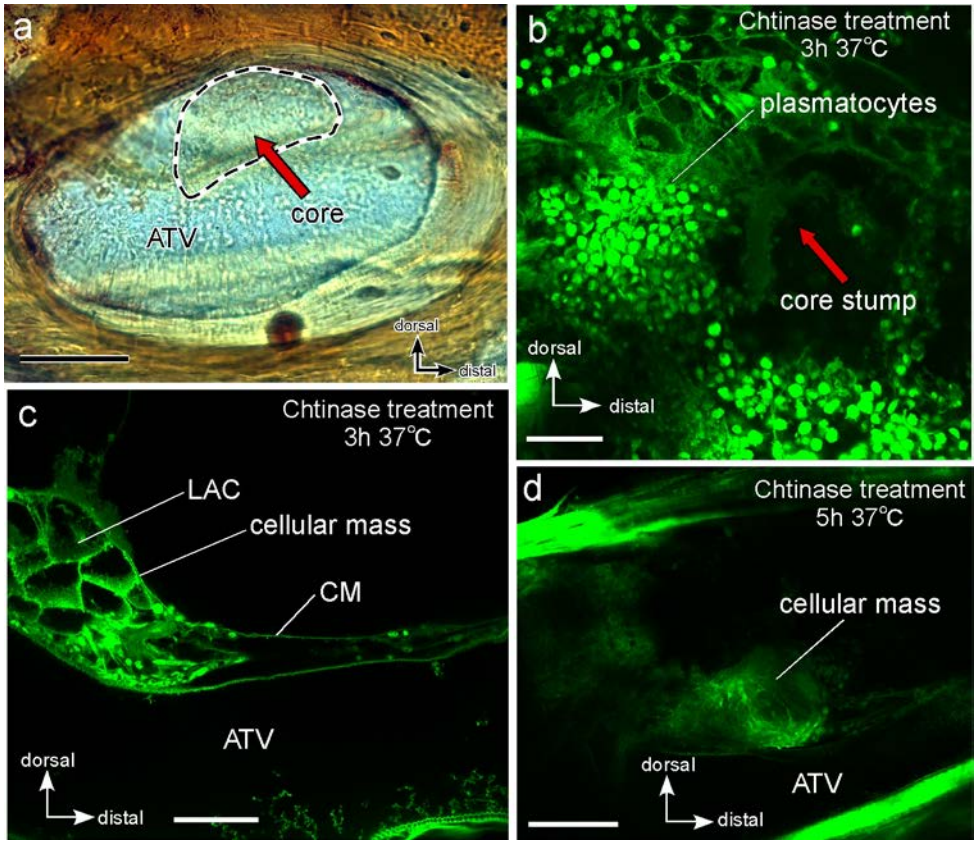

a
core
ATV
dorsal
distal
b
Chtinase treatment
3h 37°C
plasmatocytes
core stump
dorsal
distal
c
Chtinase treatment
3h 37°C
LAC
cellular mass
CM
ATV
dorsal
distal
d
Chtinase treatment
5h 37°C
cellular mass
dorsal
ATV
distal

Fig. 8

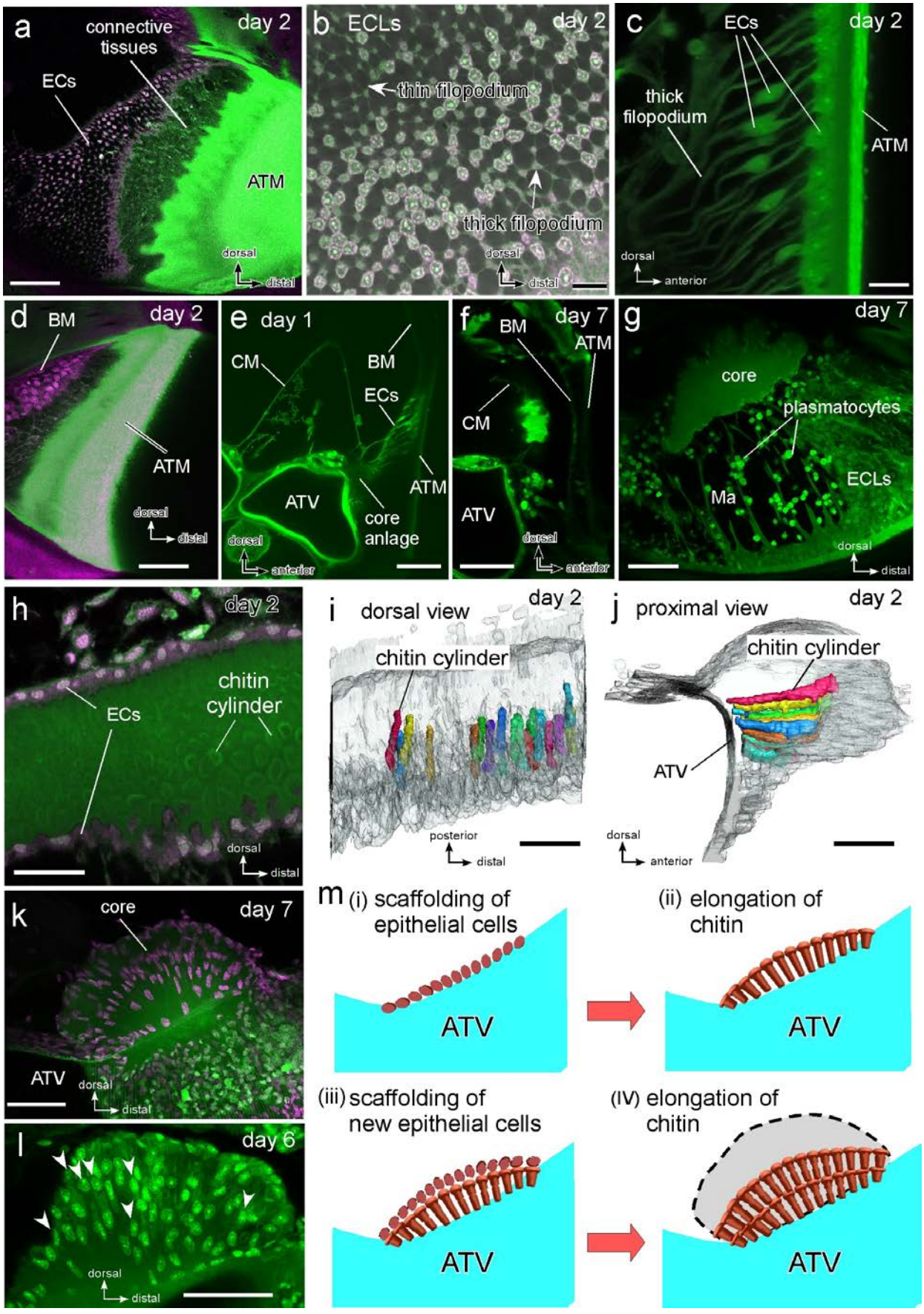

a
connective tissues
ECs
ATM
day 2
dorsal
distal
b ECLs
day 2
thin filopodium
thick filopodium
dorsal
distal
c
ECs
day 2
thick filopodium
ATM
dorsal
anterior
d BM
day 2
ATM
dorsal
distal
e day 1
CM
BM
ECs
ATM
ATV
core anlage
dorsal
anterior
f
BM
day 7
ATM
CM
ATV
dorsal
anterior
g
day 7
core
plasmatocytes
ECLs
Ma
dorsal
distal
h
day 2
chitin cylinder
ECs
dorsal
distal
i dorsal view
day 2
chitin cylinder
posterior
distal
j proximal view
day 2
chitin cylinder
ATV
dorsal
anterior
k
core
day 7
ATV
dorsal
distal
m (i) scaffolding of epithelial cells
ATV
(ii) elongation of chitin
ATV
l
day 6
dorsal
distal
(iii) scaffolding of new epithelial cells
ATV
(IV) elongation of chitin
ATV

Fig. 9

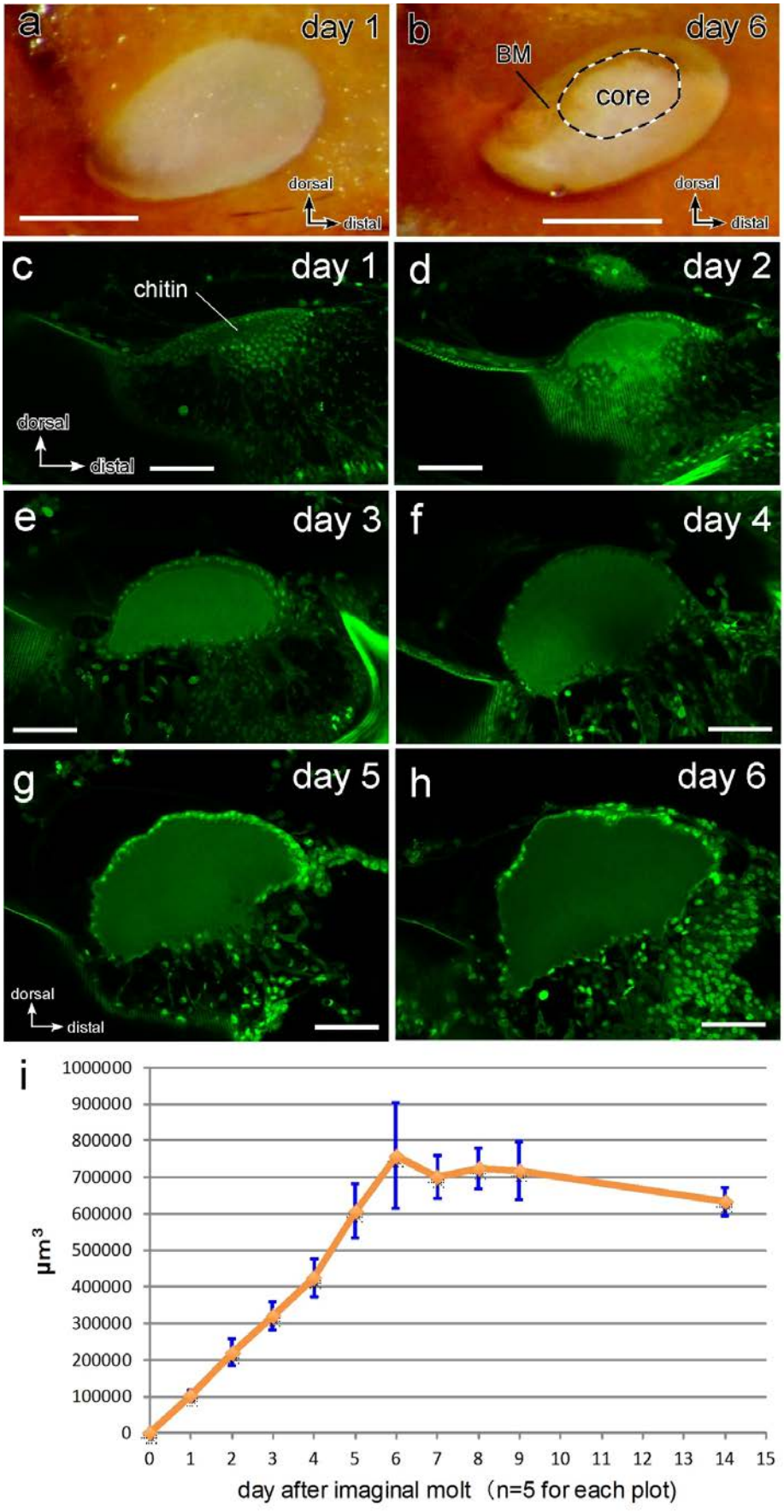
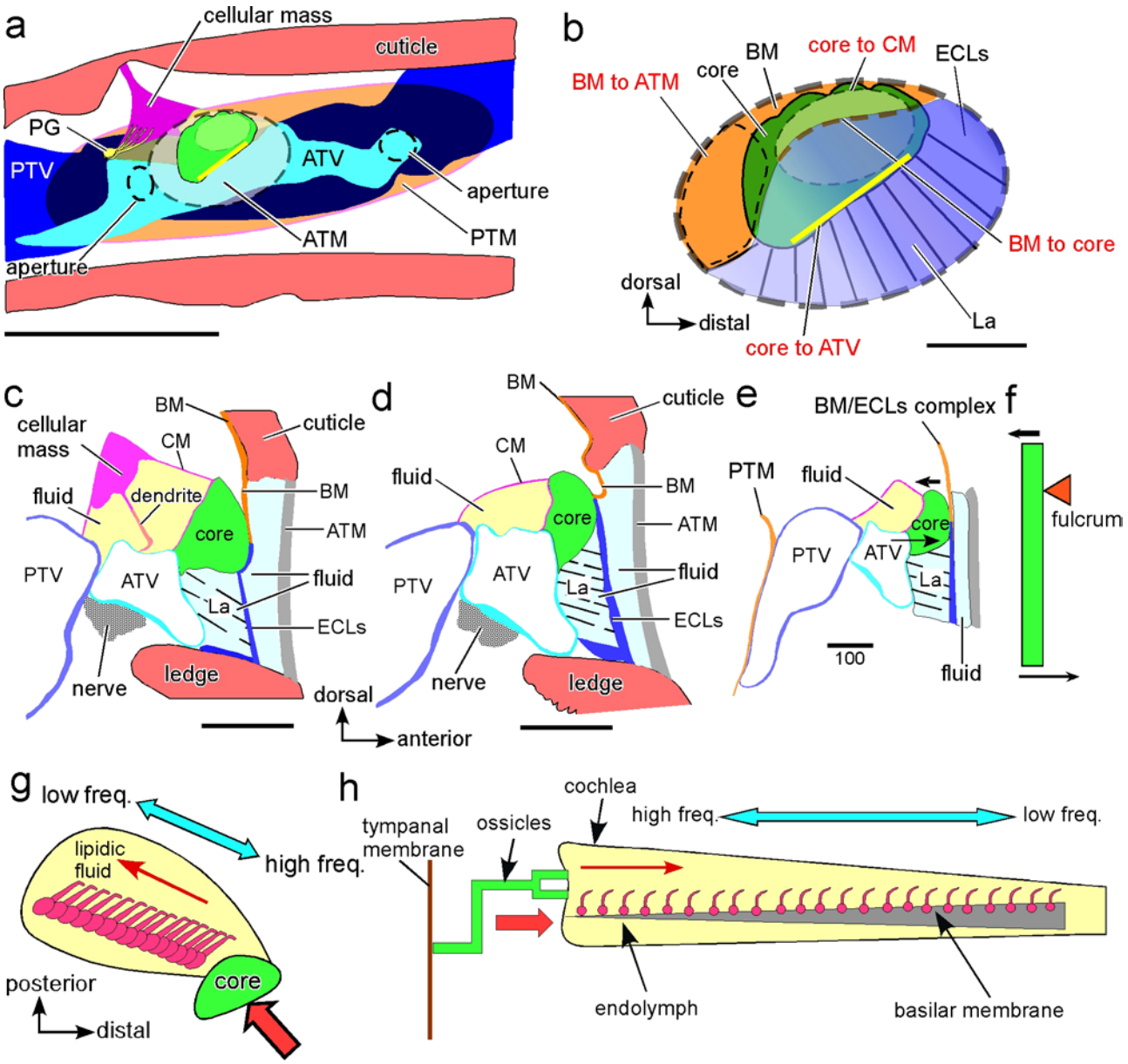

a
day 1
dorsal
distal
b
day 6
BM
core
dorsal
distal
c
day 1
chitin
dorsal
distal
d
day 2
e
day 3
f
day 4
g
day 5
dorsal
distal
h
day 6
i
µm³
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
day after imaginal molt（n=5 for each plot)

Fig. 10


a
cellular mass
cuticle
PG
PTV
ATV
aperture
ATM
PTM
aperture
b
BM to ATM
core
BM
core to CM
ECLs
BM to core
dorsal
distal
La
core to ATV
c
cellular mass
BM
cuticle
CM
fluid
dendrite
BM
core
ATM
PTV
ATV
fluid
La
ECLs
nerve
ledge
dorsal
anterior
d
BM
cuticle
CM
fluid
BM
core
ATM
PTV
ATV
fluid
La
ECLs
nerve
ledge
e
BM/ECLs complex
PTM
fluid
core
PTV
ATV
La
100
fluid
f
fulcrum
g
low freq.
high freq.
lipidic fluid
posterior
core
distal
h
cochlea
high freq.
low freq.
tympanal membrane
ossicles
endolymph
basilar membrane