

Morphological and Developmental Traits of the Binucleation of Male Accessory Gland Cells in the Benthic Water Bug, *Aphelocheirus vittatus* (Hemiptera: Aphelochiridae)

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Abstract

The male accessory glands (MAGs) in insects are pair(s) of internal reproductive organs that produce and secrete the plasma component of seminal fluid. In various insects, MAG size is important for male reproductive success because the fluid provides physiologically active substances and/or nutrients to females to control sperm as well as female reproductive behaviors. Although the MAG epithelial cells in most insect species are standard mononucleate cells, those in some insect taxa are binucleate due to incomplete cytokinesis (e.g., *Drosophila* [Fallén] [Diptera: Drosophilidae] or cell fusion (e.g., *Cimex* [Linnaeus] [Hemiptera: Cimicidae]). In the case of *Drosophila*, the apicobasal position of the two nuclei relative to the epithelial plane changes from vertical to horizontal after nutrient intake, which allows the volume of the MAG cavity to expand effectively. On the other hand, in the case of *Cimex*, the positions of the two nuclei do not change apicobasally in response to feeding, but their position relative to the proximodistal axis varies depending on the tubular/spherical organ morphology. Here, we report that the MAG of the benthic water bug *Aphelocheirus vittatus* (Matsumura) (Hemiptera: Aphelochiridae) shows binucleation in all epithelial cells. Despite the phylogenetically close relationship between *Aphelocheirus* and *Cimex*, the MAG cells in *Aphelocheirus* showed a *Drosophila*-like apicobasal change in the position of the two nuclei in response to feeding. Furthermore, the cytological processes during binucleation are more similar to those in *Drosophila* (incomplete cytokinesis) than to those in *Cimex* (cell fusion). These results indicate that the physiological role and mechanism of binucleation in MAG cells changed during the evolution of Hemiptera.

Second abstract in Japanese language

昆虫の雄性附属腺は内部生殖器官の1つであり、精液中の精子以外の成分を産生および外分泌する。様々な昆虫において、雄性附属腺の大きさはオスの繁殖成功にとって重要であることが知られており、各種の生理活性物質や栄養成分をメスに送り込むことによって、精子やメスの生殖行動を制御している。多くの種では、雄性附属腺の上皮細胞は通常の1核細胞であるが、いくつかの分類群では2核細胞であり、それは細胞質分裂の不全(シヨウジョウバ工)や細胞融合(トコジラミ)によってもたらされている。シヨウジョウバ工では栄養の摂取に伴い、頂端基底軸上の2核の配置が上皮面に対して垂直から水平へと変化し、それは雄性附属腺の内容積を効果的に拡大させるのに役立つが、トコジラミではそのような核配置の変化は見られない。今回我々は、底生性の半翅目であるナベブタムシ *Aphelocheirus vittatus* の雄性附属腺が、全上皮細胞において2核であることを報告する。ナベブタムシとトコジラミの系統進化上近縁な関係にも拘わらず、ナベブタムシの附属腺上皮細胞の2核は、摂食に伴って、シヨウジョウバ工のような頂端基底軸に対する配置変化を見せた。さらに、2核化の際の細胞学的特徴は、トコジラミ(細胞融合)よりもシヨウジョウバ工(細胞質分裂不全)に近いものであった。これらの結果は、雄性附属腺の生理的役割や2核化の機構が、半翅目の進化過程で変化したことを示している。

Key words: reproduction, male accessory gland, epithelium, binucleation, feeding

The male accessory glands (MAGs) are tubular or spherical exocrine organs of the internal reproductive system in insects that are

positionally and functionally analogous to the prostate in mammals. The MAGs produce and secrete the plasma component of seminal

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fluid, which is merged with spermatozoa during copulation and is transferred into the bursa copulatrix in females. MAG products are known to play several important roles in male reproductive success. One is the temporal activation of spermatozoa just before fertilization, which has been extensively examined in biochemical studies of the silk moth, *Bombyx mori* (Osanai et al. 1987, Nagaoka et al. 2012). The second role is the peptidergic regulation of female reproductive behaviors such as feeding, oviposition, and rejection of mating with other males, which have been elucidated in detail using the genetically amenable insect, *Drosophila melanogaster* (Chen et al. 1988, Ravi Ram and Wolfner 2007). Finally, males in some insect species, such as katydids, fireflies, and dobsonflies, transfer a massive amount of the MAG product to females as a nuptial gift that contains various nutrients to increase egg production (Gwynne 2001, Lewis et al. 2004, Liu et al. 2015). Because producing a larger amount of MAG products results in higher male reproductive success, various insects have evolved to increase their MAG size. For example, in stalk-eyed flies, the proportion of MAG size relative to body size tends to increase in response to increasing nutrient intake (Rogers et al. 2008). In harpaline beetles, a unilateral testis was lost during development, so that the vacant abdominal cavity could be occupied by MAGs (Will et al. 2005). In the case of the fruit fly *Drosophila* (Bangham et al. 2002), individuals possessing a larger MAG volume show higher reproductive capacity. The epithelia of the MAG in *Drosophila* are composed of two types of cells: the numerous polygonal ‘main cells’ and the fewer round ‘secondary cells’. All cells of both cell types are binucleate. The two nuclei change their apicobasal position from vertical to horizontal with respect to the epithelial plane, leading to higher plasticity in the apical area of each cell in order to generate a larger volume of ejaculate (Taniguchi et al. 2012, 2018; Takeda et al. 2019).

Despite the merits of cell binucleation in the MAG epithelium for increasing reproductive success, only a few insect taxa other than *Drosophila*, such as tephritid fruit flies (*Ceratitis capitata* [Marchini et al. 2003] and *Bactrocera tryoni* [Radhakrishnan et al. 2009]) and cimicid bed bugs (*Cimex lectularius* and *Cimex hemipterus*; Takeda et al. 2019), are known to have binucleated cells in their MAG epithelia. Furthermore, in the *Cimex* accessory gland, which is composed of two organs, the mesadenial gland for production and the mesadenial reservoir for storage, the apicobasal position of the two nuclei is always horizontal to the epithelial plane and does not respond to nutrient intake (Takeda et al. 2019). Instead, binucleation was predicted to contribute to forming the proper morphology of each organ; that is, the elongated shape of the binucleate cells is aligned unidirectionally to form the tubular shape of the mesadenial gland, whereas the cell shape is aligned omnidirectionally to form the spherical shape of the mesadenial reservoir. Additionally, in the case of the MAG of *D. melanogaster*, elongated binucleated cells are aligned unidirectionally among the main cells, but not in the secondary cells in local views, whereas they are placed more randomly in global views to form the weakly swollen tubular sac of the MAG of this species (Takeda et al. 2019).

These observations led us to predict that, at a glance, the physiological purposes of binucleation in the MAG epithelial cells are different between the two insect orders Diptera and Hemiptera. However, here, in a new example of a hemipteran aphelochirid benthic water bug, we show that the cytological traits of the binucleated MAG cells refute this prediction. Despite its phylogenetically close relationship to *Cimex*, *Aphelocheirus* shows *Drosophila*-like MAG binucleate cells in which the two nuclei are apicobasally overlaid under nonfeeding conditions and change their relative position horizontally in response to feeding. Under fully fed conditions, all of the epithelial cells also show an ordered array of the two nuclei relative to the proximodistal (PD) axis, which suggests that their

binucleation also contributes to the formation of the long tubular shape of the MAG in this species. Furthermore, the cytological process of binucleation in *Aphelocheirus* is different from that in *Cimex* but rather similar to that in dipteran *Drosophila*, indicating that multiple origins of binucleation exist even in Hemiptera. We thus point out that the physiological role and developmental mechanism of the binucleation of the MAG cells can change during evolution and often becomes common to phylogenetically distant taxa depending on the species-specific reproductive traits related to the appropriate accumulation and ejaculation volume of seminal fluid.

Materials and Methods

Materials and Rearing

Nymphs and adults of *Aphelocheirus vittatus* were collected from several rivers in Tochigi and Tottori Prefectures in Japan from August 2018 to March 2020 and bred in tap water with the Brazilian freshwater algae, *Egeria densa*, at room temperature (25°C). Commercially available frozen chironomid larvae (Kyorin Co., Ltd., Hyogo, Japan) were fed to the nymphs and adults of *A. vittatus* every week.

Dissection, Optical Microscopy, and Fixation of MAG

The whole reproductive organs were dissected from the abdomens in standard phosphate-buffered saline (PBS) using fine forceps (#5 Dumont) and spring scissors (#15002-08 FST) under a binocular microscope (Leica Microsystems, Wetzlar, Germany). The excised organs were photographed by a VHX-2000 digital microscope (Keyence, Tokyo, Japan). The organs were fixed in 4% formaldehyde (Nakalai Tesque, Japan) in PBS for 20 min with intermittent mixing at room temperature.

Immunofluorescence of MAG

The fixed organs were thoroughly washed in PBT (PBS containing 0.1% Triton X-100; Nakalai Tesque, Japan), and the following reagents were used for tissue staining: 4',6-diamidino-2-phenylindole (0.1 µg/ml, Sigma) was used for nuclear staining. A 1:100 dilution of rhodamine-labeled phalloidin (Life Technology) was used for F-actin staining. A 1:100 dilution of rabbit anti-pH3 antibody (Upstate Biotech) was used for the staining of phospho-histone H3 on the chromosomes in the mitotic phase. A 1:200 dilution of Alexa Fluor 647-labeled anti-rabbit IgG (Jackson ImmunoResearch) was also used. The staining was performed as previously described (Takeda et al. 2018).

Laser Confocal Microscopy

Laser confocal microscopic images of the tissue sections were obtained with a Digital Eclipse C1Si (Nikon), FV1000 or FV3000 (Olympus).

Transmission Electron Microscopy

An adult MAG was obtained by dissection as mentioned above and fixed with 2% glutaraldehyde/0.1 M phosphate buffer and subsequently with 2% OsO₄/0.1 M phosphate buffer. After fixation, a small piece of MAG was embedded in Quetol-812 resin (Nisshin-EM) and sliced into ultrathin sections of 70 nm thickness, which were stained with uranyl acetate-lead citrate. Transmission electron microscopy photographs were obtained using a JEM-1200EX (JEOL) with an 80-kV accelerating voltage.

Measurement of the Angles of the Lines Connecting Two Nuclei Relative to the PD Axis

Among the various laser confocal images in the surface view of the MAG, several parts showing the PD axis straight on were chosen for the measurement of the angles of the two nuclei. Lines connecting the centroids of the two nuclei were drawn in the numerous binucleated cells that were chosen randomly but excluding those near the outer edge regions of the MAG epithelia. The angles were measured by using the graphic software ImageJ (NIH, version 1.52a) and categorized into 18 ranges of 10-degree width (total 180 degree) in rose graphs depicted by the graphic software Rose.NET 0.10.0.

Results

Binucleation of All MAG Epithelial Cells in *A. vittatus*

Individual adult males that had been collected from wild habitats were examined for the cellular morphology of their MAG epithelia. As shown in the laser confocal image in Fig. 1A, all of the nuclei were distributed as pairs of two closely positioned nuclei. Although F-actin was not visible around the plasma membrane in the MAG epithelia in this species, the paired distribution of nuclei strongly suggested the binucleation of all epithelial cells. We thus observed transmission electron micrographs of these cells to find the plasma membrane. As a result, the plasma membrane apparently surrounding the two nuclei was detected (Fig. 1B and B'''). The development of rough endoplasmic reticulum and secretory granules also suggested active synthesis and secretion of proteins.

Changes in the Apicobasal Positioning of the Two Nuclei in Response to Feeding

We next examined the nutrient responses of the position of the two nuclei because apicobasal positional changes in the nuclei have been shown in *Drosophila* MAG cells, as mentioned above. In a nonfeeding state after adult molting (Fig. 2A), a surface view of the confocal image of the epithelia displayed a randomly spaced distribution of nuclei (Fig. 2A'), contrary to the initial observation in Fig. 1A. At this time, however, an optical longitudinal section of the confocal image showed the apicobasally overlaid positioning of two nuclei (Fig. 2A'') as seen in the nonfeeding state of the fruit fly, *Drosophila pseudoobscura* (Takeda et al. 2019). We then observed the MAG epithelium from an adult at approximately 1 mo after feeding, which induced a drastic growth in the volume of MAGs (Fig. 2B). The MAG epithelial cells showed horizontally adjacent positioning of the two nuclei (Fig. 2B' and B''), leading to an expansion of the apical surface area, which in turn induced the enlargement of the cross-sectional diameter of the MAG cavity by approximately 20 times (Fig. 2C). This response seems to be similar to that observed in *Drosophila* rather than in the bed bug *Cimex* despite the close phylogenetic relationship between *Cimex* and *Aphelocheirus*.

Stable Planar Angles of the Two Nuclei Relative to the PD Axis

We also noticed that after enough feeding, the angles of the lines connecting the two nuclei seemed to be neatly aligned with respect to the PD axis of the tubular shape of the MAGs (Fig. 3A), as previously observed in the mesadenial gland of *C. lectularius* (Takeda et al. 2019). Thus, we measured and analyzed these planar angles in 338 epithelial cells of the MAGs. Consequently, more than 90% of the

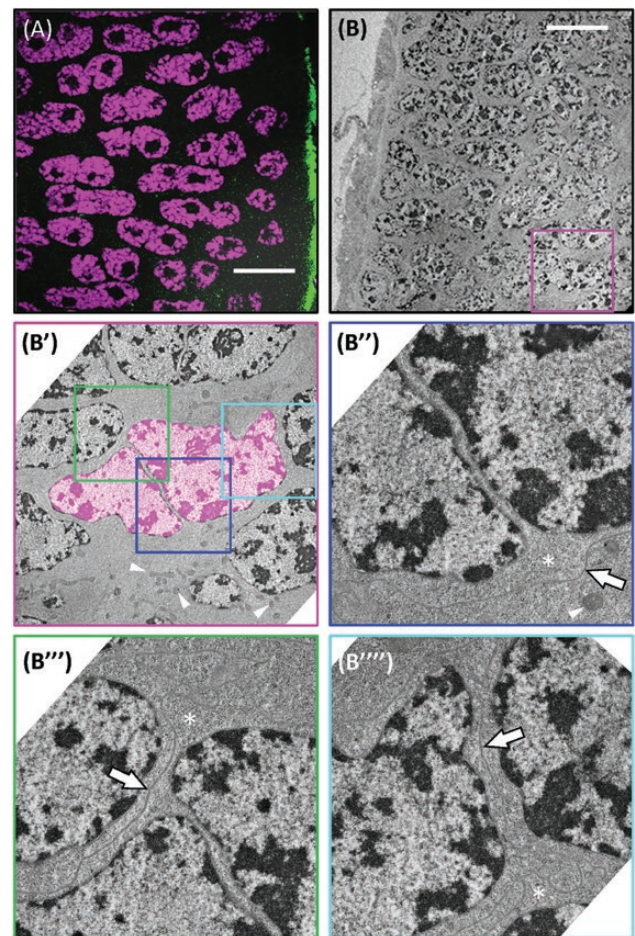


Fig. 1. Binucleation of all epithelial cells in male accessory glands (MAGs) in *Aphelocheirus vittatus*. Individuals were collected from wild habitats. (A) Laser confocal micrograph of the epithelium. Nuclei (magenta) and F-actin (green) were stained with 4',6-diamidino-2-phenylindole and phalloidin, respectively. Linear F-actin staining on the right represents circular muscles that lie at the outer edge of the MAG epithelium. A single large nucleolus exists per nucleus. Both nuclei except those in the region near the outer edge of the MAG show unevenly close positions, which suggests their binucleation. Scale bar = 20 μ m. (B) Transmission electron micrograph of the epithelium. Scale bar = 20 μ m. (B') High-magnification view of the area in the magenta square in (B). The two nuclei to be focused on are highlighted in magenta. (B''–B''''') Further high-magnification views of the areas in blue, green, or cyan squares. Note that the plasma membrane (indicated by arrows) is present between the cells but absent between the two nuclei. Arrowheads indicate secretory granules. Asterisks indicate cytoplasm filled with rough endoplasmic reticulum.

nuclei pairs showed an ordered array with more than 70° and less than 110° angles to the PD axis with an SD of 10.4°. This value indicates that the degree of ordered alignment of the two nuclei is higher than that of the *Cimex* mesadenial gland, a similar example (Fig. 3B and B', SD is 16.4). In contrast, the ordered alignment degree of the *Cimex* mesadenial reservoir, a random array example, displays a higher SD (Fig. 3C and C', SD is 37.5). These results suggest that the ordered array of the binucleate cells in *Aphelocheirus* MAG also contributes to the formation and maintenance of the tubular morphology of the MAG. Together with the abovementioned apicobasal positioning of the two nuclei, the binucleation of *Aphelocheirus* MAG cells is considered to have dual roles in both the expansion of cavity volume for seminal fluid storage and the stable formation of the tubular organ shape.

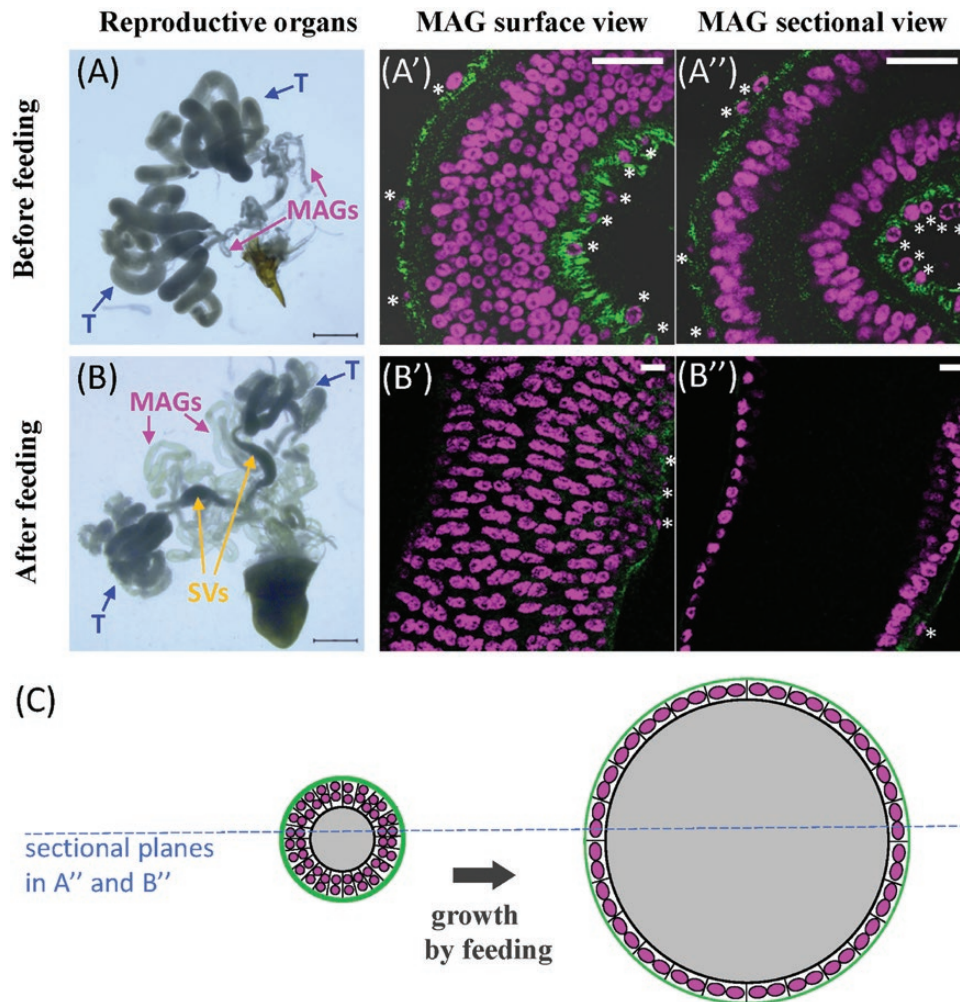


Fig. 2. Changes in the apicobasal positioning of the two nuclei in the male accessory gland (MAG) cells in response to feeding. (A and B) Whole reproductive organs. A unilateral testis and an MAG are composed of three lobes and a bifurcated tube, respectively. T, testis; SVs, seminal vesicles; MAGs, male accessory glands. (A–A'') Before feeding, after adult molting. (B–B'') Fully grown adult after feeding. In response to feeding, a remarkable expansion of the MAGs and sperm storage in seminal vesicles can be observed (B). (A' and B') Surface view of the MAG epithelium. (A'' and B'') Longitudinal section view of the same areas as in A' and B' of the MAG epithelia. Nuclei (magenta) and F-actin (green) were stained with 4',6-diamidino-2-phenylindole and phalloidin, respectively. Asterisks indicate the nuclei of the circular muscle cells that surround the epithelium. In (B'), the close positioning of the two nuclei in the epithelial cells in the same focal plane indicates the alignment of the nuclei horizontal to the epithelial plane. The elongation of each nucleus along the longer axes of the cells can also be seen. Scale bars = 500 μm in (A) and (B); 10 μm in (A'), (A''), (B'), and (B''). (C) Schematic representation of the transverse sections of the MAG before and after feeding. In terms of growth in the apicobasal direction, the volume of the MAG cavity (gray) is expected to increase approximately 20 times after growth due to feeding based on the expansion of the cavity shown in (A'') and (B'').

Cytological Events During the Binucleation of the MAG Epithelial Cells

In the *Drosophila* MAG, binucleation occurs at approximately the midpupal stage (55–60 h after puparium formation at 25°C) by synchronous entry into the last mitotic (M)-phase and subsequent incomplete cytokinesis (Taniguchi et al. 2014). On the other hand, in the *Cimex* MAG, binucleation occurs just before adult molting by synchronous fusion between adjacent cells without synchrony of the cell cycle (Takeda et al. 2019). As shown above, *Aphelocheirus* MAG displayed *Drosophila*-type apicobasally overlaid nuclei despite their phylogenetically distant relationship. Thus, we examined how binucleated MAG cells develop in *Aphelocheirus* with regard to cell-cycle synchronization and cytokinesis.

Immediately before the last molting, the epithelia of the MAG primordia showed a remarkable surge in the frequency of

phospho-histone H3-positive cells (M-phase cells) compared with that of seminal vesicles (Fig. 4A) composed of standard mononucleate cells in both the nymphal and adult stages (data not shown). This indicated that a short-term concentration of M-phase entry occurs specifically in the MAG primordia, and its mitotic index was approximately 11% (22/208). High-power imaging (Fig. 4B) showed that the mitotic cells show apically directed delamination in the epithelium as seen in standard cell division in various animal epithelia, although *Drosophila* MAG primordia do not show this type of cell movement during binucleation (Fig. 5K and L). Then, unlike in standard cell division, the MAG cells in anaphase (after karyokinesis) showed a movement to return to the epithelium without executing cytokinesis (Fig. 4C). These binucleation processes can be seen across the last molting stage, so that residual pH3-positive cells were found immediately after the last molting at a low frequency (data not shown). At this stage, it is difficult to count the

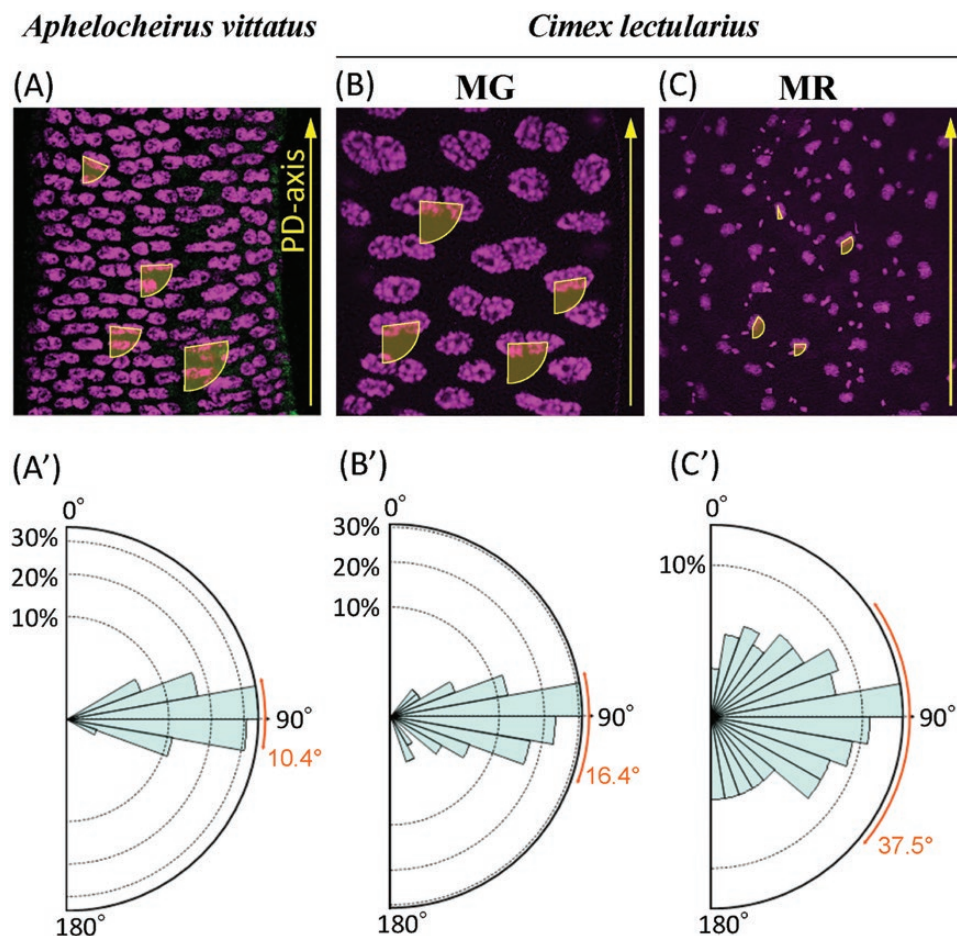


Fig. 3. Angles of lines connecting the two nuclei relative to the proximodistal (PD) axis. (A) A close-up view of the male accessory gland (MAG) of *Aphelocheirus vittatus*. Four examples of cells used to measure the angles of the two nuclei (yellow sectors) relative to the PD axis (right arrow) on the epithelial surface. (A') Rose graph representing the relative frequencies of the angles of the two nuclei in angle ranges with 10-degree intervals (338 cells in *A. vittatus*). The range with the maximal frequency is shown as the largest wedge. An orange arc and a numeral indicate the SD = 10.4°. (B) For comparison, a close-up view of the mesadenial gland (MG) in the bed bug, *Cimex lectularius*. (B') Rose graph representing 108 cells in the MG of *C. lectularius* as shown in A'. SD = 16.4° (C) For comparison, a close-up view of the mesadenial reservoir (MR) in the bed bug, *C. lectularius*. (C') Rose graph representing 210 cells in the MR of *C. lectularius* as shown in B. SD = 37.5°.

nuclear number in all cells because the cells are compacted due to poor growth. However, binucleation was confirmed in all of the epithelial cells when high-resolution images were obtained (Fig. 4D). We also noticed that there was another single small tubular organ called the 'central gland' at the anterior end of the ejaculatory duct (Fig. 4A). Although the function of this central gland is unknown, its cells show a binucleation process quite similar to that in MAG cells (Fig. 4D). The presence of the central gland is unique to this species and is not found even in phylogenetically close taxa (T. Adachi-Yamada, unpublished observation).

Discussion

Dual Physiological Significance of Binucleation of MAG Cells in Hemipteran Insects

In our previous study using the bed bug *C. lectularius*, the role of binucleation in hemipteran MAG cells was thought to be limited to the contribution to the formation of the tubular/spherical morphology of the organs. However, the present study of the benthic water bug *A. vittatus* revealed that the *Drosophila*-type alteration in the apicobasal position of the two nuclei to increase the plasticity of the

MAG cavity volume can also occur in hemipteran insects in addition to the arrayed planar polarity observed in the *Cimex* mesadenial gland. Thus, the binucleation in *Aphelocheirus* MAG cells has at least two physiological purposes. However, we do not believe that this dual purpose for binucleation is specific to this species. In our preliminary survey of various hemipteran taxa, the cytological feature of two apicobasally overlaid nuclei is sporadically observed, although its relation to feeding is unknown. Therefore, the physiological roles of MAG cell binucleation are different among various taxa and may change rapidly during evolution, as discussed below.

Possible Causes for Frequent Alterations in the Significance of Binucleation During Evolution

What are the causes of species-specific differences and common features in the roles of binucleation in MAG cells among phylogenetically distant taxa? One possible cause is the variety of requirements for epithelial plasticity among various taxa. For example, the *Cimex* accessory glands are composed of two parts, the mesadenial gland and the mesadenial reservoir, each of which works specifically for the production and storage of the exocrine substance, respectively. Thus, the mesadenial gland does not

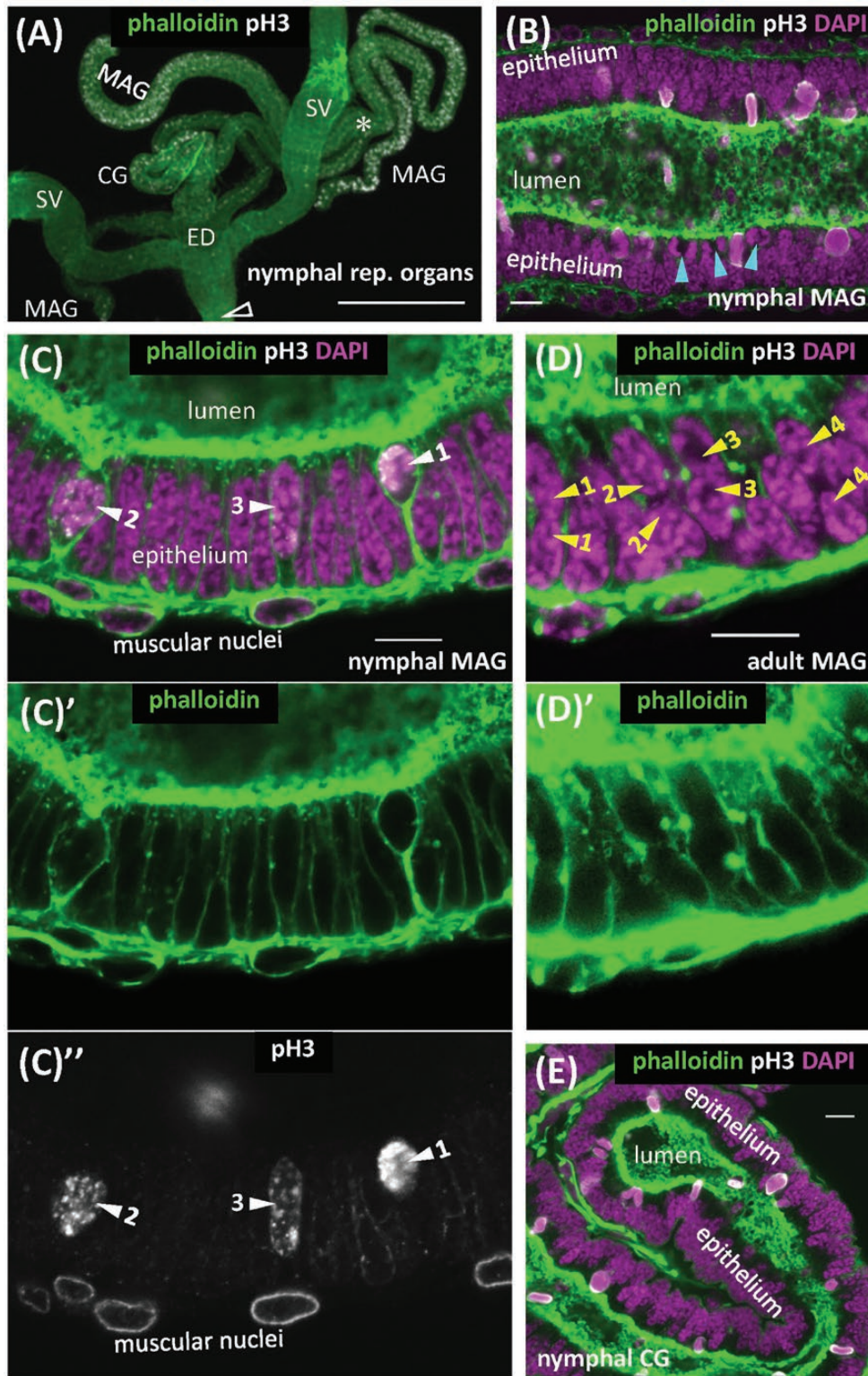


Fig. 4. Binucleation process in male accessory gland (MAG) cells of *Aphelocheirus vittatus*. (A) M-phase cells detected by anti-pH3 antibodies (white) in internal reproductive organs immediately prior to the last molting. CG, central gland; SVs, seminal vesicles; ED, ejaculatory duct. An open arrowhead indicates the division points between the MAGs and the ED. An asterisk indicates the division point of the two branches of the unilateral MAG. F-actin (green). Scale bar = 500 μ m. (B) High-power image of the MAG epithelium in (A). Apically localized M-phase cells (white) can be seen with high frequency. Cyan arrowheads indicate anaphase cells. DAPI (magenta). Scale bar = 10 μ m. (C) Apically localized anaphase cells move back to the MAG epithelium without cytokinesis (white arrowheads). Numerals indicate the predictable order of cell movement. Scale bar = 10 μ m. (C') Green channel in (C). (C'') White channel in (C). The surface of the muscular nuclei shows positive stainability with pH3 antibody even in interphase for unknown reasons. (D) Immediately after the last molting, pH3-positive cells were no longer observed in this area, and binucleate epithelial cells could be seen. Yellow arrowheads with the same numerals indicate each nucleus in the same binucleate cell. The stainability of the F-actin network underlying the plasma membrane by phalloidin weakens immediately after this stage. Scale bar = 10 μ m. (D') Green channel in (D). (E) Nymphal central gland (CG) shows a binucleation process similar to that seen in MAG (B). Scale bar = 10 μ m.

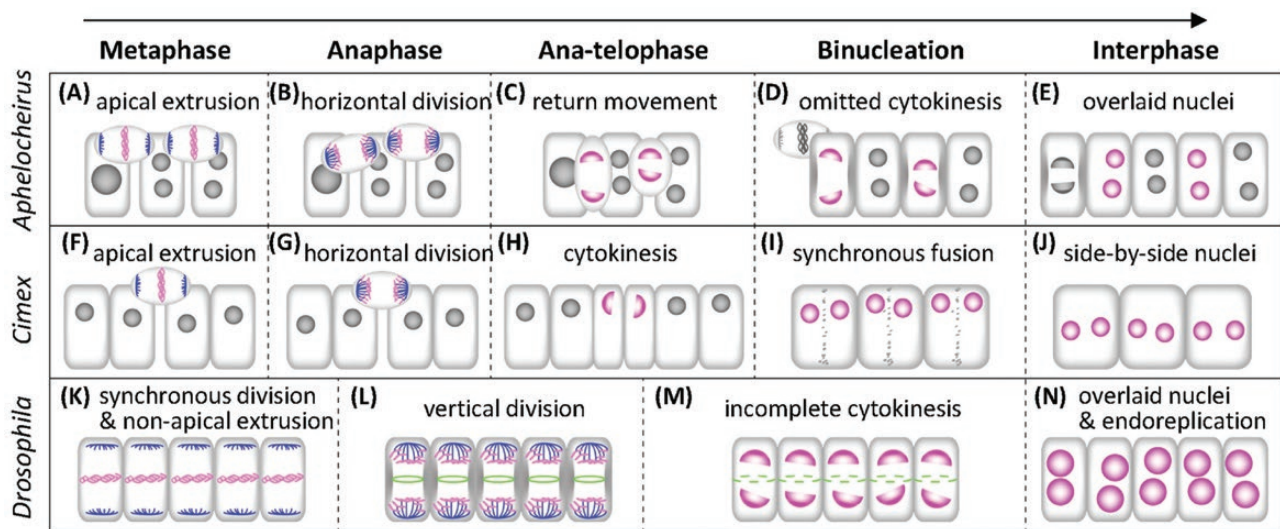


Fig. 5. Comparison of the cell morphology and binucleation processes among three insect taxa. The cell-cycle stages indicated at the top represent those in the colored cells illustrated in A–N. (A–E) *Aphelocheirus*. (F–J) *Cimex*. (K–N) *Drosophila*. (A, F, and K) Last metaphase. (B, G, and L) Anaphase. (C and H) Ana-telophase. (D, I, and M) Binucleation. (E, J, and N) Interphase. In *Aphelocheirus*, the last M-phase cells appear in the short term just before the last molting with a high mitotic index, and cytokinesis is omitted; neither residual contractile rings nor broken partitions can be observed. In *Cimex*, the cell cycles seem to be random among all cells with a low mitotic index, and the partitions between the adjacent cells disappear synchronously just before the last molting. In *Drosophila*, the cell cycles of numerous cells are synchronized at the midpupal stage, and the contractile rings disappear prior to the completion of cytokinesis. Blue = microtubules; magenta = nuclei; green = contractile ring.

Table 1. Comparison of the cytological traits of the male accessory gland with binucleated epithelial cells among four insect species

Order	Species	Organ	Angles of two nuclei relative to		Last M-phase	Reference	
			Epithelial plane	PD axis			
			Before feeding	After feeding			
Hemiptera	<i>Aphelocheirus vittatus</i>	Accessory gland and central gland	Vertical	Horizontal	Highly fixed (SD = 10.4°)	Synchronized immediately before last molting	This work
	<i>Cimex lectularius</i>	Mesadenial gland Mesadenial reservoir	Horizontal	Horizontal	Moderately fixed (SD = 16.4°) Various (SD = 37.5°)	Random and asynchronous until last molting	This work and Takeda et al. (2019)
Diptera	<i>Drosophila melanogaster</i>	Accessory gland	Angular	Horizontal	Locally fixed and globally varying	Synchronized at midpupal stage	Taniguchi et al. (2012), Takeda et al. (2019)
	<i>Drosophila pseudoobscura</i>	Accessory gland	Vertical	Horizontal	Locally fixed and globally varying	Not examined	Takeda et al. (2019)

seem to elevate epithelial plasticity to increase the storage of the MAG product. Furthermore, it has been reported that the *Cimex* mesadenial reservoir can store enough MAG product for four mating events (Reinhardt et al. 2011). If this level of storage is optimal for *Cimex* male reproduction, males should usually have excess seminal fluid for a single mating event and do not need to ejaculate a larger volume. In other cases, polyandrous females may induce a smaller ejaculation volume per mating in males, as observed in fungus-gardening ants (Mikheyev 2004) and stalk-eyed flies (Chapman et al. 2005). Furthermore, as postcopulatory males always showing mate-guarding behavior can reduce the risk of sperm competition, the size of the accessory glands may become less important (Alonzo and Warner 2000). Similarly, male-male competition to obtain mates often strongly develops sexually

attractive signals or weapons, both of which are known to trade off with spermatophore size, as shown in fireflies (Lewis et al. 2004) and dobsonflies (Liu et al. 2015), respectively. Therefore, MAG epithelium plasticity and MAG enlargement are not always absolute requisite conditions for males in all insect taxa, and they may readily change depending on the optimal species-specific behaviors in reproductive strategies.

Polyphyletic Evolution of the Binucleation of MAG Cells in Hemiptera

The remarkable difference in the binucleation process between *Cimex* and *Aphelocheirus* (summarized in Fig. 5 and Table 1), both of which belong to the same suborder, Heteroptera, was an unexpected

result. As the cell fusion and omission of cytokinesis are totally different mechanisms, both are considered to be derived from different evolutionary origins. An interesting question is how this mechanism could change. One possible explanation is based on the presence of evolutionary degeneration between the two taxa. As stated above, the importance of seminal fluid accumulation and MAG cell binucleation is highly variable depending on the species-specific reproductive strategies. Once the physiological value of binucleation declines below a certain threshold, its existence will not be under selection pressure, and the binucleate state may become unstable or lost. Subsequently, if the significance of MAG function increases again, it is possible to coopt a different mechanism. In fact, we found that numerous species in the superfamily Nepoidea, which belongs to the same infraorder, Nepomorpha, as *Aphelocheirus*, lost MAGs completely (J. Yamauchi et al., unpublished result). The match between the apicobasal nuclear positioning and the binucleation mechanism observed in *Aphelocheirus* and *Drosophila* (Table 1) seems to be a coincidence based on our survey of a wide variety of hemipteran and dipteran accessory glands (T. Adachi-Yamada et al., unpublished observation).

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