Different growth patterns of two adjacent glomeruli responsible for sex-pheromone processing during postembryonic development of the cockroach *Periplaneta americana*

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Abbreviated terms: MGC: macroglomerular complex; PA: periplanone-A; PB: periplanone-B.

Abstract (within 250 words)

In many insect species, sex pheromone is processed by specific, enlarged glomeruli in the antennal lobes of males. In the male American cockroach, two closely located glomeruli (A and B) are responsible for processing the major pheromone components (periplanone-A and -B, respectively), and these collectively form the macroglomerular complex. Afferents originating from the dorsal and ventral surfaces of the antenna tend to project to the anterior and posterior regions of the macroglomerular complex via the dorsal and ventral antennal nerves, respectively. This topographic segregation of afferents is seen only in the macroglomerular complex, and not in other glomeruli that process normal environmental odors. Using differential, anterograde dye injection into the two antennal sensory nerves, we show that the macroglomerular complex is not formed by fusion of several glomeruli, as suggested in previous studies, but that the precursors of the A- and B-glomeruli already exist in the first larval instar. The volume of afferents in the macroglomerular complex precursor increases nearly exponentially with molting times, 430-fold from the first instar to the adult. The A- and B glomeruli both undergo continuous growth during postembryonic development, but peak growth rates occur in different larval stages. The growth rate of the B-glomerulus peaked in the mid-developmental stage then declined, while growth of A-glomerulus was maintained at low level in early- to mid-developmental stages but increased greatly in later stages. These results suggest perception of sex pheromone occurs in early instars, and that PA and PB have distinct roles in different developmental stages.

Keywords: insects; olfactory afferents; macroglomerular complex; topographic organization; postembryonic development; pheromone

In insects, chemical communication by sex pheromone is crucial for mating success. In most moth and cockroach species, sex pheromone is emitted by virgin females and received by males. The American cockroach *Periplaneta americana* is an excellent model organism in which pheromone communication has been studied extensively in both neurobiology and chemical ecology [4]. Periplanone-A (PA) and periplanone-B (PB) have been isolated as major sex-pheromone components [14]. They have similar chemical compositions [14] and both can qualitatively elicit the same courtship behavior in male cockroaches [13,21]. Pheromonal components are processed by distinct PA-sensitive and PB-sensitive neurons housed in the same morphologic type of sensilla (type *single-walled B*), which are especially abundant on the antennae of the adult male [17,19]. Axons from the two types of receptor neurons each converge onto

two closely located glomeruli, collectively forming the macroglomerular complex (MGC). The remaining glomeruli (about 165), termed ordinary glomeruli [11], process normal environmental odors (e.g. food odor). Individual glomeruli are functional modules where a large number of afferents expressing cognate olfactory receptors make synaptic connections with a small number of interneurons [6]. Electrophysiological studies revealed that interneurons with dendritic arborizations restricted to the dorsal glomerulus of the MGC responded specifically to PA, while interneurons with dendritic arborizations restricted to the ventral glomerulus responded specifically to PB [2]. Although this anatomical segregation has been elucidated relatively little is known about their functional differences. Bioassay experiments using active fractions corresponding to PA and PB in the laboratory suggest that PB is responsible for long distance attraction while PA influences male behavior near the female [21,22].

Compared to holometabolous insects (e.g. moths), in which glomerular development mostly occurs during the pupal and early adult stages [7,9], cockroach glomeruli continually develop in each molting after hatching concurrent with the growth of the antenna [15,18,19]. Histological studies have suggested that the MGC of the cockroach is formed during the last third of postembryonic development by fusion of several small glomeruli [15]. Correspondingly, sex pheromone-responsive interneurons, whose dendrites cover the precursor of the MGC, have been identified in the late larval stages [20].

The most striking morphologic feature distinguishing the MGC from other ordinary glomeruli is the unique topographic organization of afferents. Whereas sensory afferents from sensilla on the dorsal and ventral surfaces of the antenna are generally intermingled in ordinary glomeruli, those from the two antennal surfaces are biased toward the anterior and posterior regions in the MGC [11,12]. Similar topographic organization is also found in the MGC of male sphinx moths [3], silk moths [1], and gypsy moths [8]. Differential labeling of the two antennal nerves enables precise tracing of afferents in the developing MGC. To gain further insight into the functional significance of PA and PB, we investigated the growth pattern and afferent organization in the MGC from the first larval instar to the adult.

Larval instars and adult male cockroaches *Periplaneta americana* with intact antennae reared in 12:12h light-dark cycle at 27˚C were used. Individuals immediately pre- and post-molting were not used. Oothecae were collected and newly hatched larval instars separated. Individuals of each developmental stage were collected from the box every day, and kept in separately. In our laboratory, the number of larval stages was usually 11. The width of the head capsule, the length of the hind tibia, and the body

length were measured in each individual as additional criteria for the evaluation of the larval age [5,10]. Sex of larval instars was readily determined by checking the morphologies of the last two abdominal sternites [16]. The dyes used for anterograde staining of antennal afferents were 10 % microruby (dextran tetramethylrhodamine with biotin, 3000 MW, D-7162, Invitrogen, Eugene, Oregon, USA) and 10% microemerald (dextran fluorescein with biotin, 3000MW, D7156, Invitrogen). There was no discernable difference between the staining patterns of the two dyes. The dissection and staining protocol is described in Nishino and Mizunami [12]. Briefly, the distal cut-ends of two parallel antennal nerves in the right antenna were placed either together in a tapered glass electrode containing microruby or separately into two tapered glass electrodes containing either microruby or microemerald. The single-dye labeling was used to analyze the volume of afferents in the MGC, and the double-dye labeling was used to observe topographic segregation of afferents originating from the two antennal surfaces. Differential labeling of the two antennal nerves in cockroaches younger than 5th instars was technically too difficult. Hence, only microruby staining data is presented for these individuals.

The brain, removed from the head capsule, was fixed in a 4% formaldehyde saline solution for 4h at 5˚C, dehydrated in an ascending ethanol series, and then cleared in methyl salicylate. The cleared brain was viewed anteriorly using a confocal laser-scanning microscope (LSM5 Pascal, Carl Zeiss, Jena, Germany). Optical sections (1~1.5 μm) of the MGC were reconstructed three-dimensionally with *Amira* software (Mercury Computer Systems, Berlin, Germany). In the present study, the PA- and the PB-processing regions, revealed by electrophysiological studies [2], are referred to as A-glomerulus and B-glomerulus, respectively, as these two regions are not fused but are spatially segregated like other glomeruli. The border between the two glomeruli was readily detectable in serial sections in all larval and adult stages (see Supplementary data files B-G). The volumes of afferents in A- and/or B-glomeruli were measured using the function of *Amira* software. The thick nerve trunks innervating the glomerulus from the lateral side were not considered as afferents for volume measurements. Mean values derived from two animals (except for first instar in which data was obtained from one animal) were plotted for each larval stage. The body axis was adopted as the reference line against which the orientations of the glomeruli are described.

In the cockroach, sensory neurons originating from sensilla on the dorsal surface of the antenna send axons to the dorsal nerve, while those on the ventral surface send axons to the ventral nerve (Fig.1a). The two nerves have almost equal thicknesses in the first larval instar and this is maintained during development [19]. During postembryonic

development, the number of sensory axons increases in proportion to the number of newly-emerged sensilla that are added to the two antennal surfaces [18, 19]. Accordingly, glomerular growth in the antennal lobe (AL, Fig. 1a) parallels the increase in incoming olfactory afferents [15].

The MGC is located on the antero-lateral edge of the antennal lobe and is characterized by its large size relative to the spherical, ordinary glomeruli (Fig. 1b). The MGC in the adult comprises the A-glomerulus located antero-dorsally (A, Fig. 1b) and the B-glomerulus located postero-ventrally (B, Fig. 1b). The number and location of ordinary glomeruli and related sensory tracts was relatively consistent from the first larval instar to the adult [15]. This feature enabled us to use surrounding structures as reliable landmarks for identification of the MGC precursor. The MGC precursor and about seven ordinary glomeruli (outlined by a broken line, Fig. 1c) are innervated by a single sensory tract, diverging dorso-laterally from the antennal nerve near the entrance of the antennal lobe (white arrow head, Fig. 1c). The MGC precursor is located most ventrally among glomeruli innervated by this sensory tract. Regardless of ages of larval instars, the location of MGC precursor was generally: 1) postero-ventro-lateral to an identifiable glomerulus (g1, Fig. 1c-f,h), 2) antero-lateral to a sensory bundle (white arrow, Fig. 1d-f) projecting dorsally to innervate a fan-shaped glomerulus (g2, Fig. 1d-f), and 3) antero-medial to a glomerulus characterized by thick afferents (g3, Fig. 1d-f). The precursors of A- and B-glomeruli were initially detectable in the first larval instar (A,B, Fig. 1d,g). In the first to fourth larval instars, the A- and B glomeruli were even smaller than other ordinary glomeruli (Fig. 1c,d; Supplementary data file A). In these early instars, the A-glomerulus was located just laterally to the B-glomerulus (Fig. 1g,h; Supplementary data files B, C), but progressively migrated more dorsally in later instars (Fig. 1i-k; Supplementary data files D-G).

The volumetric analysis of afferents in the MGC precursors revealed that the growth rate of MGC was nearly exponential during postembryonic development; the volume of afferents in the MGC increased 430 fold (386 for A-glomerulus; 455 for B-glomerulus), from ca. 1280 μm^3 in the first larval instar to ca. 553200 μm^3 in the adult (Fig. 2a, see suppl. Table 1). This increase is exceptionally large compared to ordinary glomeruli in which about 14-fold increases in volume occur from the first instar to adult [15].

From the first to the fifth larval instars, afferent growth rates of A- and B-glomeruli were similar (Fig. 2b), although initial volume of afferents in the B-glomerulus was about twice as great as that of the A-glomerulus (Fig. 2a). From the sixth instar, the A and B-glomeruli growth rates diverged. Growth rate of the B-glomerulus between the fourth and ninth moltings increased, peaking with a 4-fold increase at the sixth molt,

before returning to roughly pre-fifth molt growth for the rest of the development (Fig. 2b). In contrast, the growth rate of the A-glomerulus was maintained at a lower level (about 1.1~1.5x) from the first to the seventh instars, then increasing to about two fold in the eighth to tenth instars (Fig. 2b). The most drastic increase of afferents in the A-glomerulus (about 4.1) occurred at the imaginal ecdysis (Fig. 2b). The earlier peak growth of the B-glomerulus against the A-glomerulus resulted in a low proportional volume of the A-glomerulus in the MGC during the seventh to ninth instars (Fig. 2c; Supplementary data file E). The start of the divergent growth rates of the A- and B glomeruli coincided with the lateral to dorsal migration of the A-glomerulus with respect to the B-glomerulus suggesting that the new afferents were added almost exclusively to the lateral side of the B-glomerulus [12].

The three-dimensional reconstructions of the MGC (Fig. 3) in which afferents from the dorsal nerve (magenta) and ventral nerves (cyan) were differentially labeled, revealed that the formation of the topographic organization of afferents is also linked to the different growth patterns of the A- and B-glomeruli. The border between the afferents from the two antennal surfaces resides in the center of the MGC viewed medially (indicated by broken line, Fig. 3). In general, the afferent terminations from the two antennal surfaces became more segregated, and the demarcation between them more prominent, in later developmental stages (Fig. 3). At the fifth instar, the axons running in the dorsal and ventral nerves were already segregated toward anterior and posterior regions in the lateral sensory tract (Fig. 3a). Reflecting this, afferent terminals tended to be biased toward anterior and posterior sides of the B-glomerulus, although intermixture of afferents was evident (Fig. 3a). Such segregation of afferents was not evident in the A-glomerulus (Fig. 3a). The segregation of afferents in the A-glomerulus became evident from the eighth larval instar (Fig. 3d), which coincided with the increase in its growth rate (Fig. 2b). The topographic organization of afferents in A- and B-glomeruli was close to maturation in the tenth instar (Fig. 3f), as this is similar to the afferent organization in the adult [12].

Our study revealed two important findings. Firstly, the precursor of the MGC is already formed in the first larval instar (Fig. 1). Second, the sub-compartments of the MGC, A- and B-glomeruli take different developmental processes (Figs. 2, 3), despite their close proximity and the similar composition of chemicals that they process [14]. In the hawkmoth, glomerulus development depends on the arrival of a subset of sensory axons, and this occurs at different times for different glomeruli during the pupal stage [9]. However, two adjacent male-specific macroglomeruli (called “cumulus” and “toroid”) have similar glomerular volumes and grow at similar rates, at least in their late

developmental phases [7].

We found no evidence to support the hypothesis that several glomeruli emerge in late larval instars and fuse to form the glomerular complex [15]. In the present study, the two glomeruli were readily identifiable from specimen to specimen irrespective of larval stage (Supplementary data files B-G). In the first to about sixth larval instars, the sizes of the A-and B glomeruli are similar to those of ordinary glomeruli and their relative positions differ from that of the adult (Fig. 1). It is possible that these glomeruli were overlooked in early larval instars in the original study as it was performed by tracing glomeruli from serial sections of the brain using light microscopy, a technically challenging task [15].

The existence of an MGC precursor in the first larval instar supports the possibility that sex pheromone may be perceived by early larval instars. So far, electroantennogram (EAG) studies show that male-specific responses to sex pheromone are recorded only in later larval instars [10]. However, this does not mean there is a lack of sensitivity to sex pheromone in early larval instars, because the volume of pheromone-sensitive afferents below 7th instars appears to be too small (less than one twentieth compared to the adult) to induce sufficient EAG responses to rise above experimental noise levels (Fig. 2). In the adult male, a long *sw B* sensillum containing one PA-sensitive neuron and one PB-sensitive neuron, is responsible for the detection of sex pheromone [17,19]. Importantly, whereas about half of long *sw B* sensilla are newly-formed at the imaginal ecdysis, the other half are derived from the shorter type *sw B* sensilla of the nymphal antenna [19]. As the shorter type *sw B* sensilla exist in all larval stages [18,19], this sensilla type is likely to participate in detection of sex pheromone in larval instars. The different growth patterns of A- and B-glomeruli indicate that different numbers of sensory axons supply the corresponding glomeruli at different timing during postembryonic development. This could occur at least via either addition of afferents from pheromone-receptive sensilla containing only PA or PB-sensitive neurons, or addition of afferents from sensilla containing different numbers of PA- and PB sensitive neurons in each molting. Morphological and physiological characterization of the shorter type *sw B* sensilla on larval antennae is required.

One might speculate that morphological development of the MGC is closely linked with physiological function. Both A- and B-glomeruli grow nearly exponentially during postembryonic development (Fig. 2a). However, a closer look at their growth rates revealed that the B-glomerulus grew more quickly in the mid-developmental stage while maximum growth of A-glomerulus was concentrated in the last third of the development (Fig. 3b). This suggests a requirement for PB detection in larval instars

and a more specific need for PA in adult life. The latter possibility is in good agreement with the finding that PA is utilized by adult males to orient precisely the source of the pheromone (i.e. female) in closer range [21,22]. This function seems to be necessary only for sexually mature adults. It would be interesting if PB contributes not only to mating but also to conspecific aggregation typical of cockroaches [4]. Bioassay experiments could be used to investigate the action of PB on the behavior of larval instars.

Finally, our study shows that the topographic segregation of afferents based on the two antennal surfaces becomes more evident in later larval instars (Fig. 3). This segregation occurs in parallel to the increase in pheromone-receptive afferents from greater numbers of sensilla on the circumference of the antennae. We suspect that afferent fibers originating from newly emerged sensilla on the two antennal surfaces are evenly distributed to the anterior and posterior sides of the existing glomerular complex, and that positional information of sensilla on growing antennal surfaces are represented consistently in the MGC without rearranging sensory terminals. This assumption is supported by the finding that afferents from the dorsal and ventral antennal surfaces tend to be intermingled in the center but are spatially more segregated toward both the anterior and posterior ends of the MGC, especially in later instars (Fig. 3e,f) and adults [12]. The functional significance of this topographic segregation of pheromone-sensitive afferents is unclear because the distance between these two antennal surfaces is assumed to be negligibly small relative to the time difference when odor molecules hit the opposing antennal surfaces (thickness of the antenna in the adult = 400 μm). So far, interneurons whose dendrites are confined to the anterior or posterior regions of glomeruli have not been identified. Further work on the physiological and morphological properties of pheromone-sensitive interneurons will be needed to elucidate how somatotopic organization seen in the MGC is utilized in the central nervous system.

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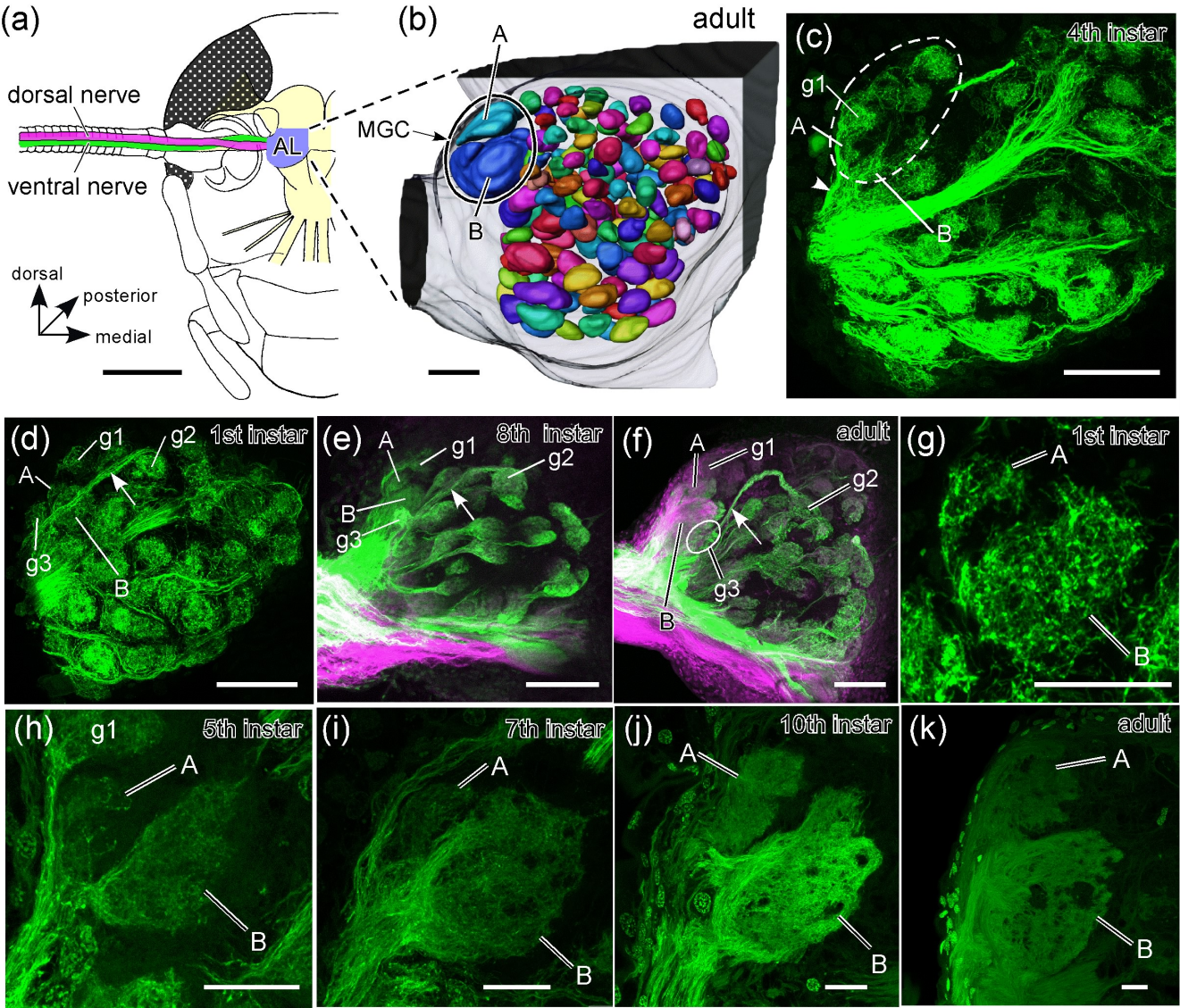
Figure Legends

Fig. 1. The macroglomerular complex (MGC) in the adult male cockroach and its precursors in different-aged larval instars, viewed anteriorly. (a) The position of the dorsal and ventral antennal nerves and the antennal lobe (AL) of the adult brain. (b) The AL contains one voluminous MGC and about 165 ordinary glomeruli. The MGC is located near the entrance of the antennal nerves. The MGC comprises the dorsally-located A-glomerulus and ventrally-located B-glomerulus. (c) In larval instars and the adult, the MGC and several ordinary glomeruli (outlined by a broken line) are innervated by a sensory tract diverging antero-laterally from the primary sensory tract (white arrowhead). In c, e and f, some ordinary glomeruli (g1-3) and a sensory tract (white arrow) are used as "landmarks" to identify A- and B glomeruli (see Supplementary data file A). In e and f, the dorsal and ventral antennal nerves, differentially labeled, are shown in magenta and cyan (see a). g-k: Confocal stacks of the central region of the MGC at different larval stages. Note that lateral to dorsal shift of the A-glomerulus occurs in the seventh instar (i) and enlargement of the A-glomerulus occurs in the tenth instar (j). Scale bars=1mm in (a); 100 μm in (b,e,f); 50 μm in (c,d); 20 μm in (g-k).

Fig. 2. Volumetric analyses of afferents in the A-glomerulus (broken line), B-glomerulus (solid line), and MGC (dotted line) during postembryonic development. (a) Log-linear plot of the increase of afferent volumes in A-glomerulus, B-glomerulus, and MGC. (b) Relative growth plots. The volume ratio of afferents before and after molting in A-glomerulus and B-glomerulus. (c) Relative volume of afferents in the A- and B glomeruli versus the whole MGC (A+B). N=2 for each point. See supplementary table for values.

Fig. 3. Three-dimensional representations of the MGC in the fifth (a), sixth (b), seventh (c), eighth (d), ninth (e), and tenth (f) larval instars. In each column, the MGC is viewed anteriorly (left) and medially (right). Afferents originating from the dorsal and ventral surfaces of the antenna are labeled with magenta and cyan, respectively (see Fig. 1a). The marginal region of afferents from the two antennal surfaces is indicated by a broken line in the medial views of MGCs. From the fifth larval instar, axons of sensory neurons originating from the two antennal surfaces were segregated before reaching the MGC. Afferents from the dorsal and ventral antennal surface tended to project to the anterior and posterior regions of the B-glomerulus, respectively, initially in the fifth larval instar

(a). This tendency is progressively more prominent in later instars. In the A-glomerulus, such segregation was not detectable until the seventh instar (c). The segregation of afferents based on the two antennal surfaces is almost completed in the tenth instar (f). Scale bars= 10 μm in (a,b); 50 μm in (c-f).


(a)
dorsal nerve
ventral nerve
AL
dorsal
posterior
medial
(b)
adult
A
MGC
B
(c)
4th instar
g1
A
B
(d)
g1
g2
1st instar
A
g3
B
(e)
A
g1
8th instar
g2
B
g3
(f)
A
g1
adult
g2
g3
B
(g)
1st instar
A
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(h)
g1
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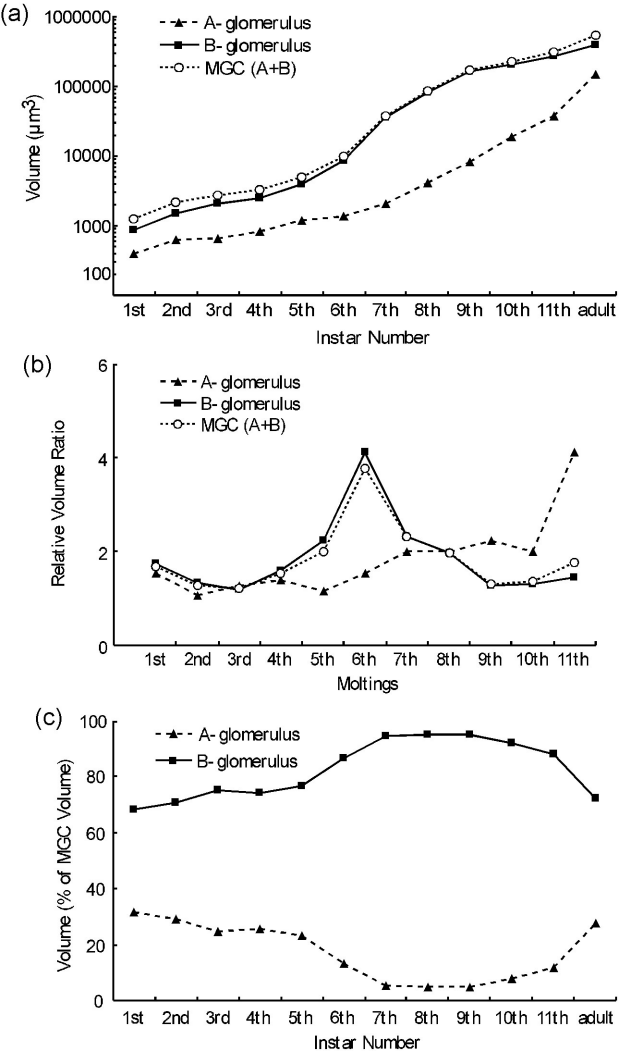
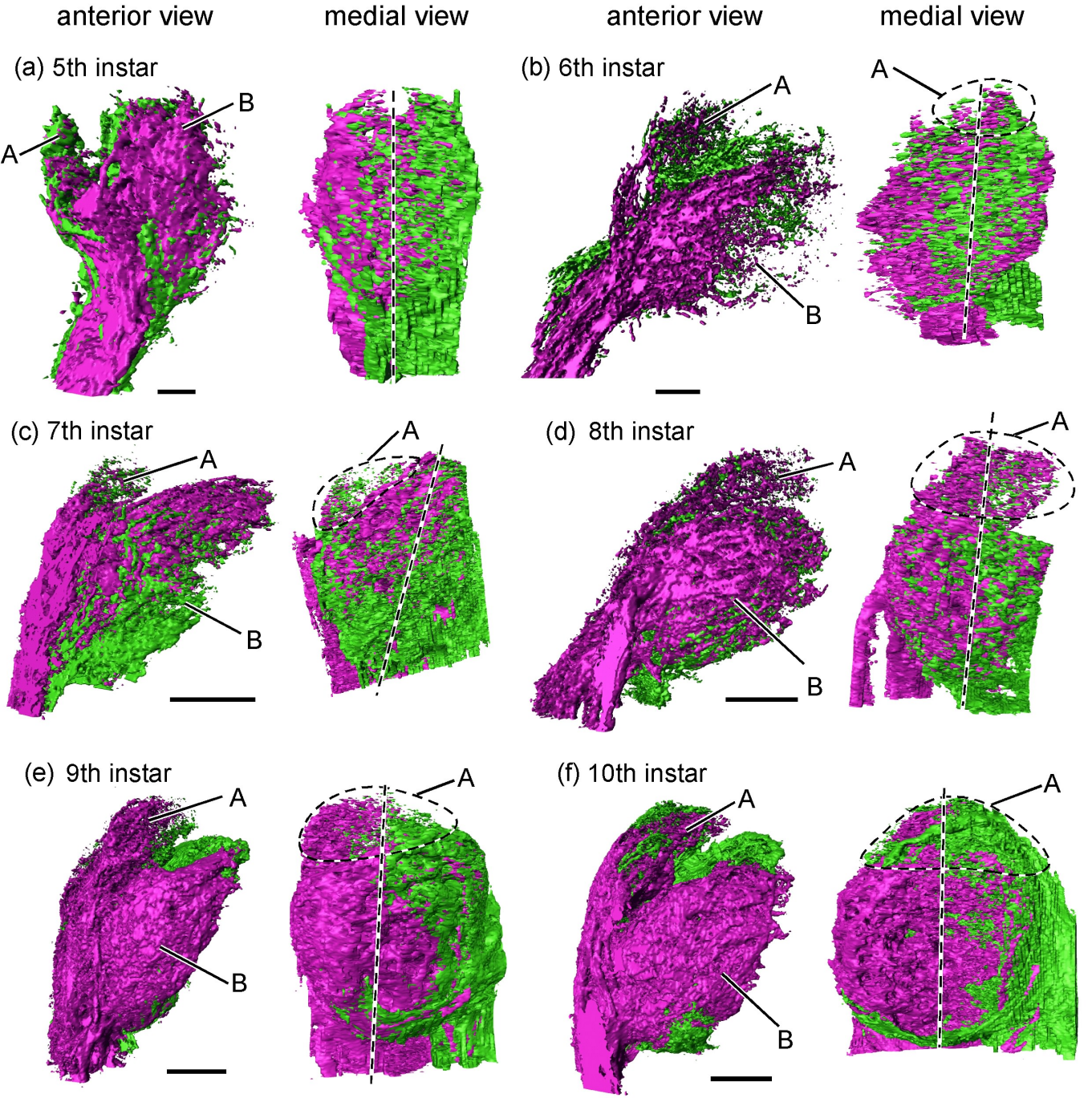
A
10th instar
B
(k)
adult
A
B


(a)
A- glomerulus
B- glomerulus
MGC (A+B)
Volume (µm3)
1000000
100000
10000
1000
100
1st 2nd 3rd 4th 5th 6th 7th 8th 9th 10th 11th adult
Instar Number
(b)
A- glomerulus
B- glomerulus
MGC (A+B)
Relative Volume Ratio
6
4
2
0
1st 2nd 3rd 4th 5th 6th 7th 8th 9th 10th 11th
Moltings
(c)
A- glomerulus
B- glomerulus
Volume (% of MGC Volume)
100
80
60
40
20
0
1st 2nd 3rd 4th 5th 6th 7th 8th 9th 10th 11th adult
Instar Number


anterior view
medial view
anterior view
medial view
(a) 5th instar
A
B
(b) 6th instar
A
B
A
(c) 7th instar
A
B
A
(d) 8th instar
A
B
A
(e) 9th instar
A
B
A
(f) 10th instar
A
B
A