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Sexually Dimorphic and Isomorphic Glomeruli in the Antennal Lobes of the Sphinx Moth *Manduca sexta*

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Abstract

Antennal lobes of adult male and female *Manduca sexta* were compared in order to investigate the nature and extent of sexual dimorphism of the primary olfactory center of this lepidopteran species. Complete identification of the glomeruli led to the conclusion that all female glomeruli have homologous male counterparts. Thus, there is no sex-specific glomerulus present in one sex and absent in the other. Sexual dimorphism (i.e. glomeruli present but morphologically different in males and females), however, does occur in the three glomeruli composing the male macroglomerular complex. The female homologs of this complex consist of two previously identified 'large female glomeruli' and one newly identified normal-sized glomerulus. The lateral and medial large female glomeruli are interpreted to be homologous to the first two macroglomerular-complex glomeruli—the cumulus and toroid 1. The third male component, the toroid 2, was tentatively identified with a normal-sized spheroidal glomerulus of the female, called here the 'small female glomerulus'. The 60 'ordinary' glomeruli that make up the rest of the glomerular neuropil were found to be homologous in males and females, with the exception of two anomalous (or uncertain) glomeruli. Some variations in relative position and size observed among those glomeruli suggest a diffuse, quantitative kind of sexual dimorphism.

Introduction

The first-order olfactory centers in the brains of most advanced animals exhibit characteristic compartments of neuropil, called glomeruli. These centers receive their input from olfactory sensory organs and send their output to higher brain centers. Detailed comparisons of these centers and their glomeruli have revealed striking similarities in their organization that suggest similar processing of odor information in animals belonging to different phyla (Hildebrand and Shepherd, 1997). The neural organization found in the giant sphinx moth *Manduca sexta* is fairly typical of that found in insects in general [see reviews in (Rospars, 1988; Rospars and Chambille, 1989; Hildebrand and Shepherd, 1997; Anton and Homberg, 1999)]. Each olfactory receptor cell (ORC) has its cell body and dendritic branches housed within a sensillum borne by the antenna and projects its axon to a single glomerulus in the ipsilateral antennal (olfactory) lobe (AL) (Camazine and Hildebrand, 1979; Tolbert and Hildebrand, 1981; Christensen *et al.*, 1995). The glomeruli also receive the neurites of AL neurons of three distinct types: local interneurons characterized by wide-field arborizations extending to most or all of the glomeruli and two types of projection (output) neurons, uniglomerular and multiglomerular ('wide-field'). Each uniglomerular projection neuron has its dendritic arborizations limited to a single glomerulus and an axon projecting to the protocerebrum. Each wide-field projection neuron has its arborization in a few glomeruli and an axon projecting to the protocerebrum. A few neurons from other regions of the CNS also terminate in the glomeruli (Homberg *et al.*, 1988). Almost all synapses in the AL are located in the glomeruli (Tolbert, 1989; Malun, 1991; Sun *et al.*, 1997).

In several insect species the glomeruli are anatomically identifiable units. This was first proposed for cockroaches (Rospars *et al.*, 1979; Chambille *et al.*, 1980) and then extended to moths (Rospars, 1983), including *Manduca* (Rospars and Hildebrand, 1992), honey bees (Arnold *et al.* 1985; Flanagan and Mercer, 1989), fruit flies (Stocker, 1994; Laissue *et al.*, 1999), and a vertebrate, the zebrafish (Baier and Korsching, 1994). This anatomical identity is believed to underlie the functional specificity of glomeruli, an increasing number of studies suggesting that the quality of an odor stimulus is represented spatially across the glomerular array in both insects and vertebrates (Jourdan *et al.*, 1980; Shepherd, 1981, 1993; Lancet *et al.*, 1982; Rospars, 1983; Rodrigues, 1988; Hansson *et al.*, 1991; Scott, 1991; Kauer and Cinelli, 1993; Ressler *et al.*, 1994; Christensen *et al.*, 1995; Hildebrand, 1995, 1996; Mombaerts, 1996;

Hildebrand and Shepherd, 1997; Joerges *et al.*, 1997; Vickers *et al.*, 1998).

The ALs are sexually dimorphic. Several types of sexual differences occur that concern either (i) the spatial locations or the volumes of glomeruli that are homologous in males and females, which is sexual *dimorphism* in the strict sense, or (ii) the presence of glomeruli that exist in one sex but not in the other, called here sexual *specificity* [see review in (Rospars, 1988)]. The best known examples of sexual difference of the ALs are the macroglomerulus of cockroaches (Jawlowski, 1948; Boeckh *et al.*, 1977; Rospars and Chambille, 1986) and the macroglomerular complex (MGC) of moths (Bretschneider, 1924; Matsumoto and Hildebrand, 1981; Rospars, 1983; Koontz and Schneider, 1987; Hansson *et al.* 1991, 1995; Ochieng' *et al.*, 1995; Todd *et al.*, 1995; Anton *et al.*, 1997; Berg *et al.*, 1998), which apparently are present only in male ALs. In moths, the MGC correlates with the presence of long, type-I trichoid sensilla that are found mainly or exclusively on the male antenna. In male *Manduca*, most of these sensilla house two ORCs, each of which is sensitive to one of the two key components of the conspecific female moth's sex pheromone (Kaissling *et al.*, 1989; Keil, 1989; Lee and Strausfeld, 1990). Each of these two ORCs projects to one of the two main MGC glomeruli, called the cumulus and toroid 1 (Hansson *et al.*, 1991; Christensen *et al.*, 1995, 1996). The function of the third, recently discovered glomerulus of the MGC, called toroid 2, is still unknown (Strausfeld, 1989; Homberg *et al.*, 1995; Heinbockel and Hildebrand, 1998). Projection neurons with arborizations in the MGC have been studied in detail (Kanzaki *et al.*, 1989; Christensen and Hildebrand, 1994; Heinbockel and Hildebrand, 1998) [see also reviews in (Christensen and Hildebrand, 1987; Homberg *et al.* 1989; Boeckh *et al.*, 1990; Hansson *et al.*, 1991; Hildebrand *et al.*, 1992; Boeckh and Tolbert, 1993; Hildebrand, 1996; Christensen, 1997)]. Recently, two relatively large and early-developing glomeruli in the dorsolateral region of each AL of female *Manduca*, have been described (Rössler *et al.*, 1998, 1999) and are called 'large female glomeruli' (LFGs; J. Roche King, T.A. Christensen and J.G. Hildebrand, in preparation). The LFGs are not found in males.

In this paper, we address three questions. (i) Are the glomeruli of the MGC unique to males or do they have homologs in females? (ii) Similarly, are the two LFGs unique to females or do they have homologs in males? (iii) Are there other cases of sexual differences among the 'ordinary' glomeruli that make up the rest of the glomerular neuropil of the AL? These questions are aimed at distinguishing between sexual dimorphism and sexual specificity, which are distinct from functional and evolutionary points of view.

Materials and methods

Animals and histology

Manduca sexta (Lepidoptera: Sphingidae) were reared as described previously (Sanes and Hildebrand, 1976). Brains excised from newly eclosed adult males and females were fixed in alcoholic Bouin's solution, embedded in Paraplast plus (Monoject Scientific, St Louis, MO) and sectioned at 8 μm in the horizontal or frontal plane. Sections were stained by Bodian's silver protargol technique (Gregory, 1980). The male brain used in this study was previously described in Rospars and Hildebrand (Rospars and Hildebrand, 1992), where it was called Ms2.

Measurements and computer reconstructions

The data were acquired and processed as described previously (Rospars, 1983; Chambille and Rospars, 1985; Rospars and Hildebrand, 1992). Except for 3-D reconstruction software, all programs were custom made. The main steps can be summarized as follows.

Delineation of profiles

Photomicrographs of serial sections were printed at magnifications of ×200 or ×190. Profiles of glomeruli, AL cell groups and tracts, and main protocerebral structures (in horizontal sections only) were traced from the photographs onto tracing paper. Each glomerulus was given a provisional name n (see below).

Superimposition of tracings

Successive tracings were superimposed and perpendicular axes were drawn corresponding to the sagittal and horizontal (on frontal sections) or frontal (on horizontal sections) planes.

Digitization and data reduction

For the males, the profiles were traced on a digitizing tablet, and the center and diameter d of each glomerulus were calculated from these data after storage in a database. For the females, the centers of glomeruli were estimated by eye and the diameters of glomeruli were obtained by averaging three measurements (depth, width and length). The reduced data (n, x, y, z, d) were stored as ASCII files.

Representations

The data were represented graphically as 2-D and 3-D reconstructions. Because of the different sectioning planes in the male and female brains used in this study, direct comparison of photomicrographs was not feasible. Identification of homologous glomeruli in different ALs relied entirely on computer reconstructions. In 2-D reconstructions, spheres representing the glomeruli and cell groups were projected on the six sides of a cube aligned with the standard coordinate axes, i.e. sides perpendicular respectively to the mediolateral X, posteroanterior Y, and ventrodorsal Z axes. For 3-D reconstructions, the same data were written in VRML (Virtual Reality Modeling Language) (Bell *et al.*, 1995) format, displayed with CosmoPlayer (Silicon Graphics, Inc., http://cosmosoftware.com) under Netscape Navigator and printed via Corel Draw.

Naming of glomeruli

Conventions that were defined previously (Rospars and Hildebrand, 1992) were used in this study. Before identification, provisional names were used; for example Msf2-R14 refers to glomerulus with provisional number 14, in the right (R) AL of female (f) number 2. After identification a final number preceded by letter G was given to each glomerulus; thus G16, for example, refers to the same glomerulus in any AL of *Manduca*, whatever its side and sex. G numbers are sorted from dorsal to ventral, so that small numbers refer to glomeruli located dorsally and larger numbers, to ventral glomeruli. The names used here are consistent with those reported in Rospars and Hildebrand (Rospars and Hildebrand, 1992).

Results

Analysis of males

Macroglomerular complex

The two main glomeruli of the MGC, the cumulus (C) and toroid 1 (T1), stand outside the array of the other glomeruli, near the entrance of the antennal nerve into the AL. These glomeruli are represented as transparent objects in computer reconstructions, either individually (Figure 1) or as if forming a single mass (Figures 2–4). The second toroid (T2) is included in the approximately spheroidal array of ordinary glomeruli. This is an elongated, horseshoe-shaped mass (Heinbockel and Hildebrand, 1998), extending in a plane perpendicular to the axis of the antennal nerve and to the horizontal sectioning plane used. In our previous work (Rospars and Hildebrand, 1992), T2 was divided into three glomeruli (G1, G11 and G24). Although the separation between G11 and G24 appears now to be an artefact due to the unusual shape and bending of this mass, G1 was a glomerulus distinct from the mass G11–G24 in one AL of both individuals studied (right Msm1 where the parts were separated by a gap; left Msm2 where they were adjacent). In Figures 1–4, T2 is represented as a set of three adjacent spheres.

Ordinary glomeruli

Sixty ordinary glomeruli were found in all male ALs studied. Most of them are spheroidal, with diameters varying between ~50 and 100 μm. Among the largest are G20, in a posterolateral location close to T2, and G64, the so called LPO glomerulus, in a ventral location, in which terminate the axons from the labial-palp pit organ (Kent *et al.*, 1986).

Intra- and interindividually anomalous glomeruli

Some glomeruli do not fit in this pattern. They are found in one or both ALs of a single individual and have no clearly recognizable homologs in the other brains (or in the contralateral AL). Two examples of the second category of asymmetric glomeruli, the very small G65 and G66, and one of the first category of symmetric, interindividually anomalous glomeruli, the medium-sized G38, were found in one of the males studied (Rospars and Hildebrand, 1992). For this reason these glomeruli are believed to be supernumerary and can be considered as minor (G65, G66) or more conspicuous (G38) developmental mistakes. The ALs

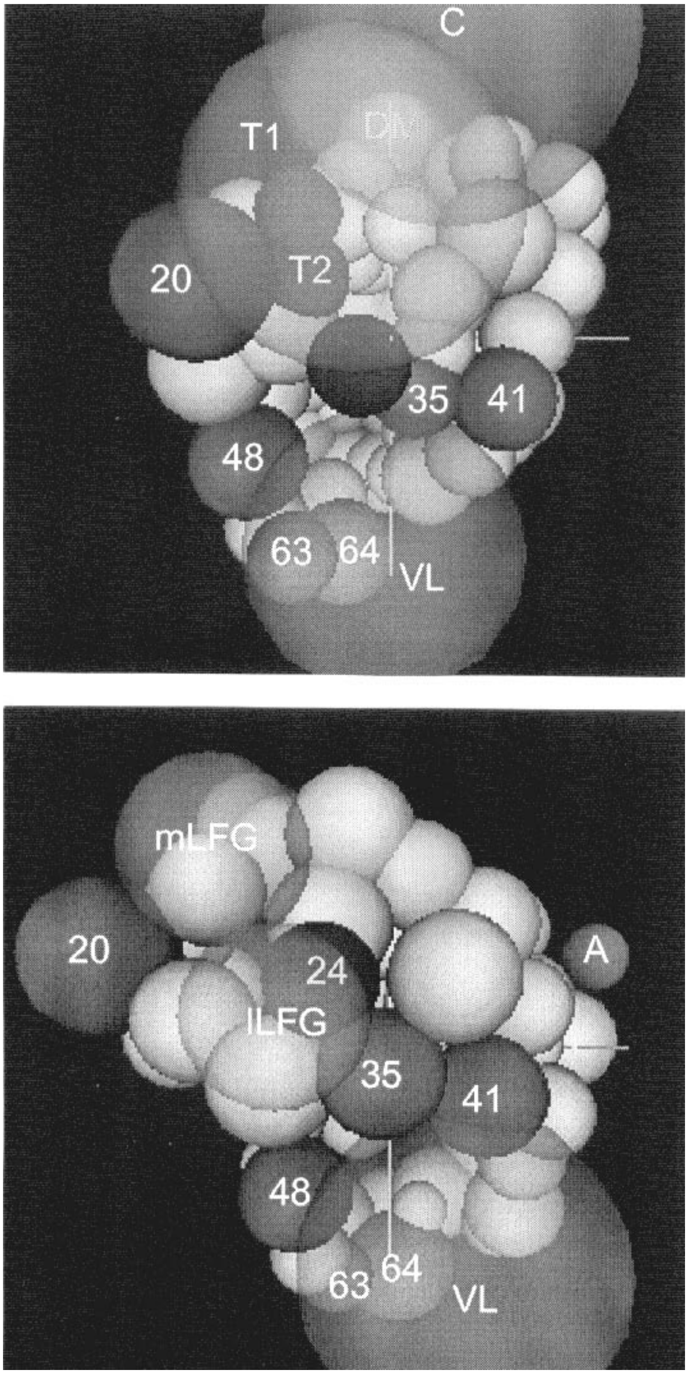

Figure 1 Three-dimensional reconstructions of ALs of a male and a female adult *Manduca*. Individual glomeruli of the male macroglomerular complex are shown separately. Conventions similar to those of Figures 2–4 are used to represent glomeruli and cell groups.

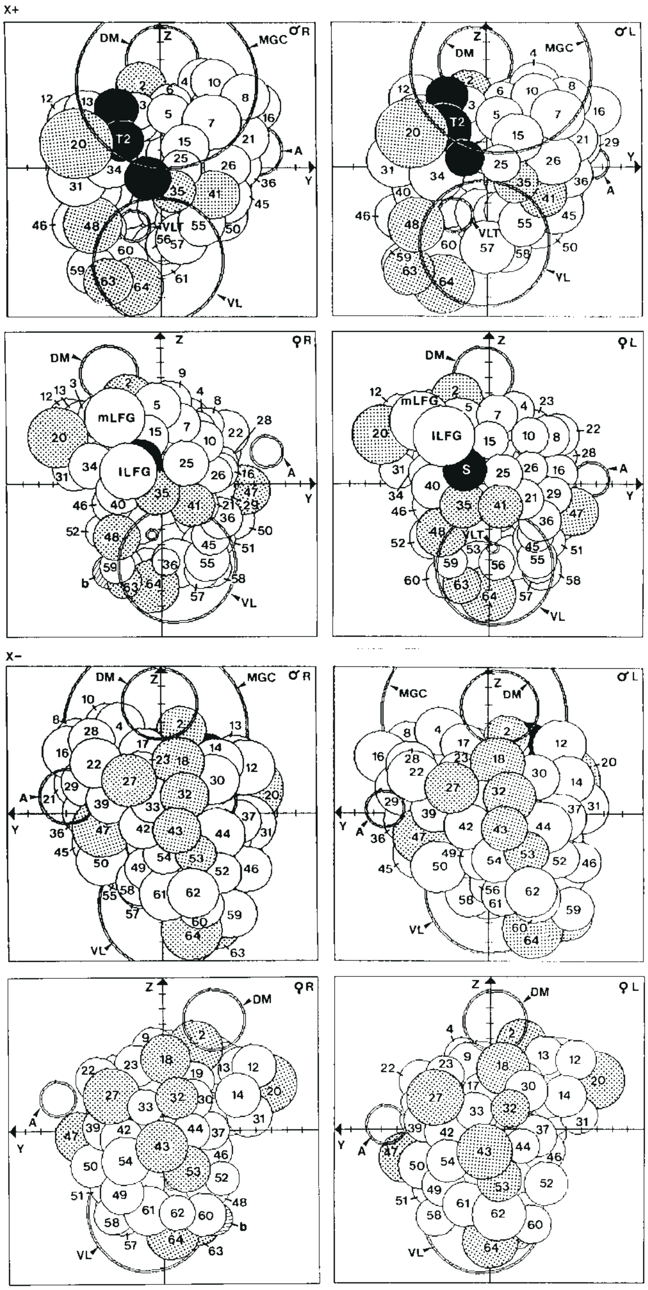

Figure 2 Lateral (X+) and medial (X–) 2-dimensional reconstructions of ALs of a male (top row) and a female (bottom row) adult *Manduca*. ALs are viewed from outside by an observer located on the opposite sides of the mediolateral X-axis. For each point of view the right (R) and left (L) ALs of each individual (male and female) are shown (four drawings). The two bulkier glomeruli of the male macroglomerular complex (C and T1) are shown as a single mass (MGC) with double rings, as if transparent. The third glomerulus (T2) of the MGC is shown as a group of three black spheres; its presumptive female homolog (S, the small female glomerulus) is also shown as a black sphere. The most easily identifiable glomeruli, including the two largest ordinary glomeruli (G20 and G64, the LPO-glomerulus), are shown in grey. Glomerulus 'b' (hatched), visible in X+ and X– of the female right AL, is anomalous. Note that numbers G1, G11, G24, G38, G65 and G66 are not used in these drawings. A, anterior group of AL neurons; DM, dorsomedial group of AL neurons; VL, ventrolateral group of AL neurons; VLT, VL tract; SEG, subesophageal ganglion. The length of the square frame is 400 μm.

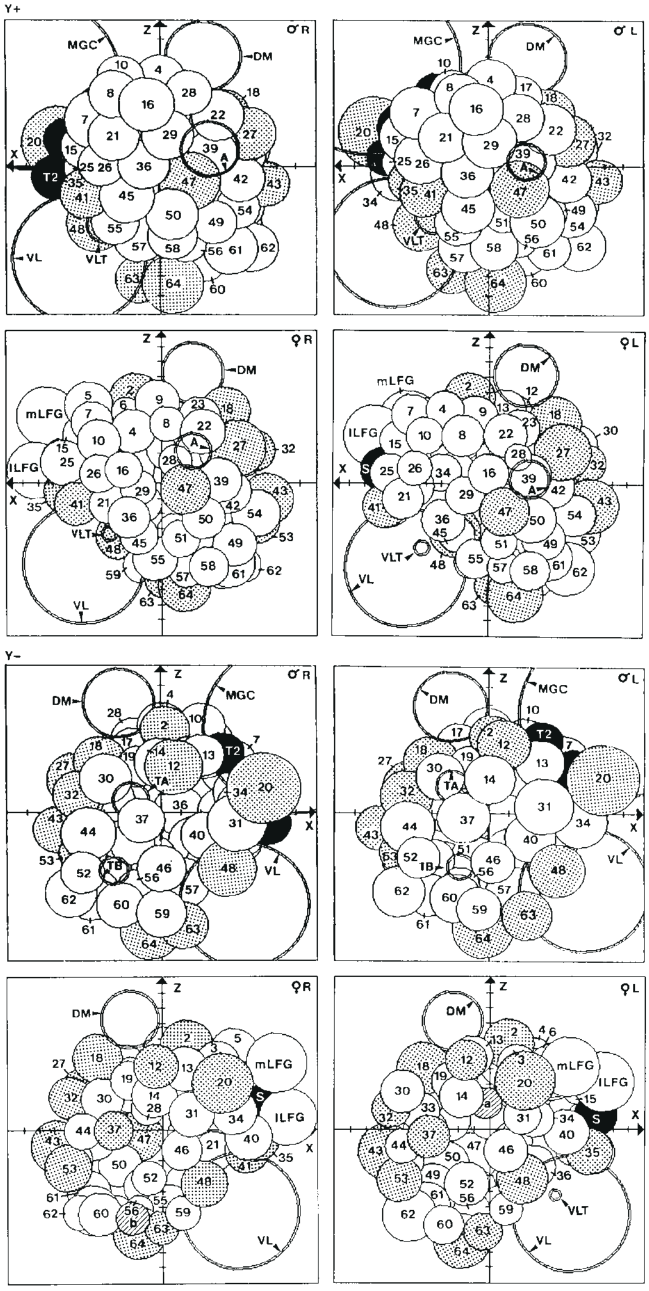

Figure 3 Anterior (Y+) and posterior (Y–) views of ALs. Same representation as in Figure 2. Hatched glomeruli 'a' (Y–, female left AL) and 'b' (Y–, female right AL) are anomalous.

of the individual shown in Figures 1–4 did not exhibit such anomalies.

Analysis of females

Frontal sections of the ALs of an adult female *Manduca* are shown in Figures 5 (anterior sections) and 6 (posterior sections).

Female glomerular complex

Two large glomeruli (the LFGs), close to the entrance of the

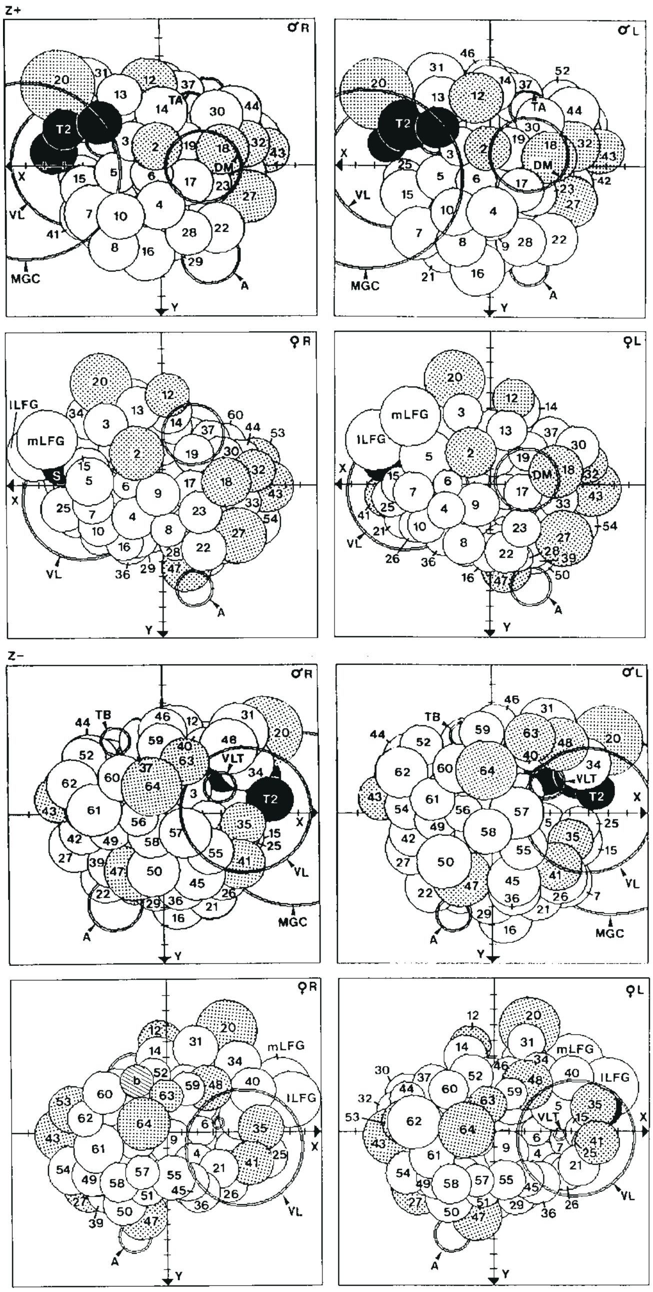

Figure 4 Dorsal (Z+) and ventral (Z–) views of ALs. Same representation as in Figure 2.

antennal nerve, are the 'female-specific glomeruli' described by Rössler *et al.* (1998). These large female glomeruli are approximately the same size (~92–100 µm in diameter) and among the largest glomeruli in the AL. One is close to the lateral pole of the AL and is called here the 'lateral large female glomerulus' (lLFG; Figure 6A–D), and the other is more medial in the dorsoanterior sector ('medial large female glomerulus', mLFG; Figure 6A–D). Whether other glomeruli are associated with the large female glomeruli in a female glomerular complex is considered in the section on male–female comparison, below.

Ordinary glomeruli

As in the male, all other glomeruli are spheroidal, or sometimes oblong, with diameters ranging from ~45 to 100 µm. Sixty-one glomeruli (not including the two large female glomeruli) were found in both right and left ALs of the female brain analysed in this study. Again, among the largest are G20 (Figure 6E,F) and G64 (Figure 5E). The homologous pairs were determined by a careful comparison of the relative positions of the glomeruli in the six side views of the 2-D reconstructions (Figures 2–4). A unique, consistent solution was reached in a relatively straightforward way with very few ambiguous pairs.

Intraindividually anomalous glomeruli

A single glomerulus, in the left AL close to the posterior pole (denoted 'a' in Figure 3 Y–), had no recognizable homolog in the right AL. With a diameter of 52 µm, this is one of the smallest glomeruli. Another problematic mass, also small (54 µm), was found only in the right AL, close to the ventral pole (denoted 'b' in Figure 4 Z–, see posteromedial quadrant), but its glomerular status is uncertain.

Male–female comparison

Invariant ordinary glomeruli

Almost the same number of ordinary glomeruli (i.e. not including the sex-specific complexes or the anomalous glomeruli) was found in the two brains represented in Figures 2–4, i.e. 60 in the male and 61 in the female. Equality of total numbers, however, does not mean identity of individual glomeruli, as evidenced by the existence of anomalous glomeruli. Therefore, a stepwise process was followed to demonstrate their identity and to locate the supernumerary glomerulus of the female ALs. First, some distinctive male glomeruli were identified also in the female ALs; all of them are shown in grey in Figures 2–4. This is obvious for G20 and the labial-palp pit organ glomerulus (G64) and its neighbors G63 and G48 (Figure 6A; see lateral view Figure 2 X+). The same clear homology can be demonstrated for some other glomeruli, such as the series G27, G32, G43 and G53 in the medial views (Figure 2 X–), G47 in the anterior view (Figure 3 Y+), G12 and G37 in the posterior views (Figure 3 Y–), G2, G18, and again G27 in the dorsal views (Figure 4 Z+), as well as G35 and G41 in the ventral views (Figure 4 Z–). However, the identification of the other glomeruli is less straightforward and must be based on a detailed analysis using the criterion of relative position. These systematic comparisons, gradually extended, showed that the same local patterns exhibited by male glomeruli can also be found in the female ALs. In this way, a consistent pairing emerged. A complete correspondence, with no ambiguous glomeruli, could be established between the two lists, and the supernumerary female glomerulus could be identified as a glomerulus adjacent to the lateral large female glomerulus (denoted S in Figures 2–4, see below). The results of these analyses are summarized in

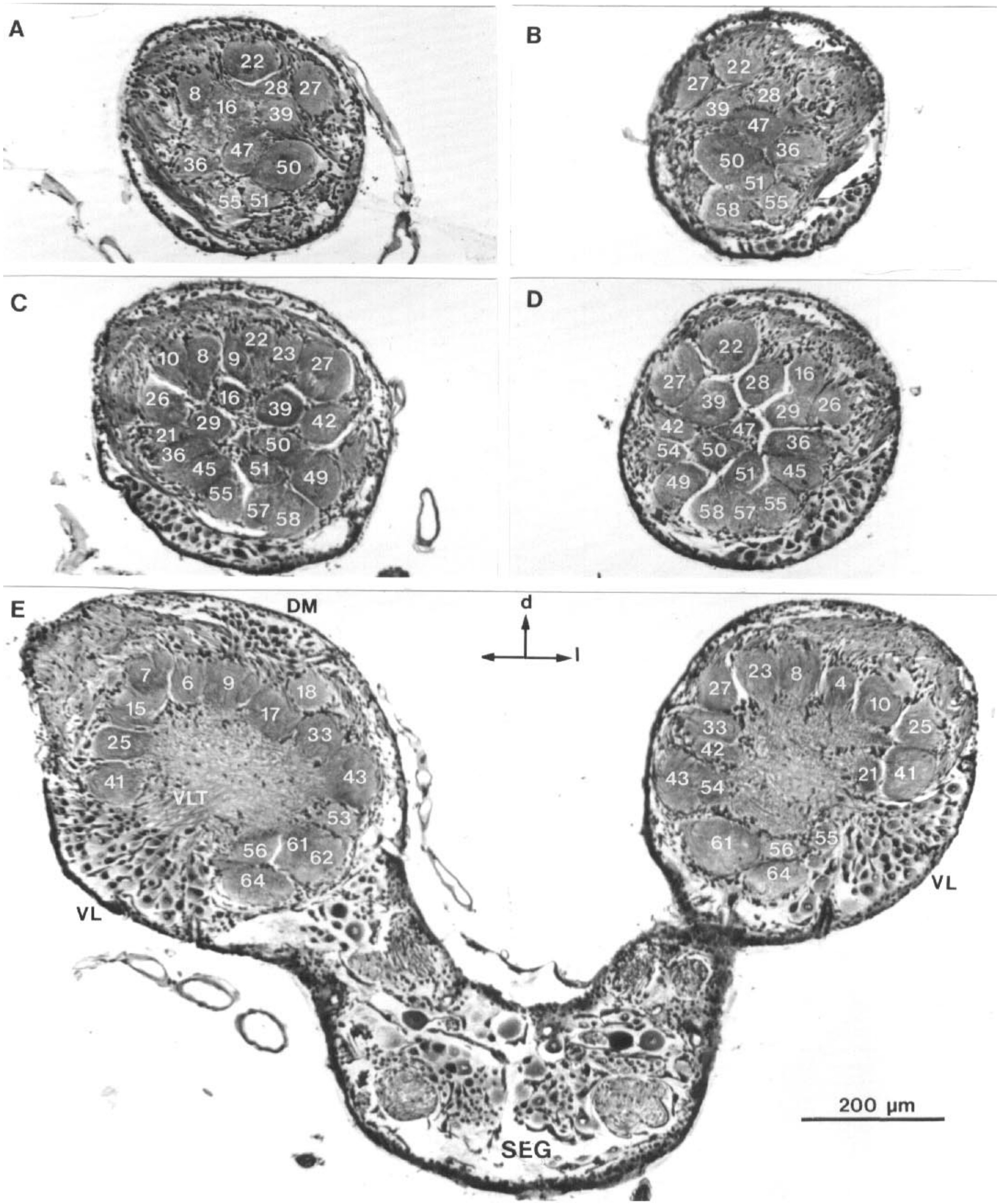

Figure 5 Anterior aspects of the left **(A, C, E)** and right **(B, D, E)** female ALs, showing bilateral symmetry of the glomerular organization and one of the largest glomeruli G64 (LPO-glomerulus) in E. Sections Nos 9 (A), 13 (B), 14 (C), 16 (D) and 23 (E). DM, dorsomedial cell group; VL, ventrolateral cell group; VLT, VL tract; SEG, subesophageal ganglion; d, dorsal; l, lateral.

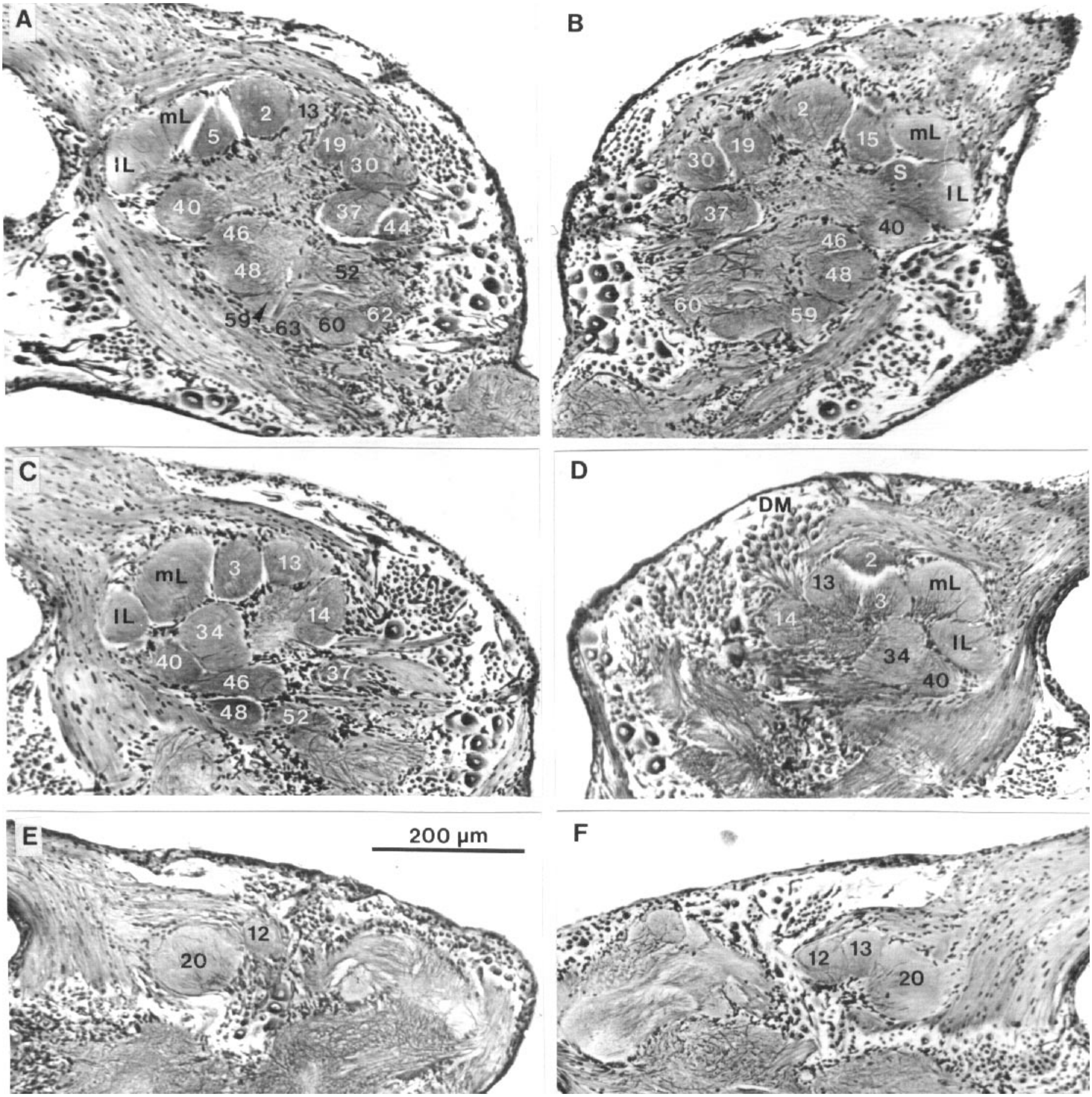

Figure 6 Posterior aspects of the left **(A, C, E)** and right **(B, D, F)** female ALs showing the female homologs of the male macroglomerular complex, lL (lFDG), mL (mFDG) and S (SFDG), and one of the two largest regular glomeruli G20 (E, F). Sections Nos 33 (A), 36 (B), 37 (C), 41 (D), 45 (E) and 50 (F). Same abbreviations and orientation as in Figure 5.

Figures 2–4, where homologous glomeruli are given the same numbers in the four ALs shown.

Sexually dimorphic glomeruli

The qualitative homology between ordinary glomeruli of both sexes sheds new light on the nature of the relationship between the male and female sexually dimorphic glomeruli. From their position alone, one is led to suggest that the cumulus and first toroid in the male's MGC are the homologs of the large female glomeruli in the female's AL, although there is no conclusive evidence that the cumulus is the homolog of the lateral large female glomerulus or that the first toroid is the homolog of the medial large female glomerulus, or vice versa. It is tempting to identify the second toroid in the male's MGC with the right–left couple left over in the preceding comparison, which is adequately located (see Figure 6B). In fact, there are no alternatives, because all other glomeruli in the neighborhood were paired in both males and females. This third dimorphic glomerulus can be called the 'small female glomerulus' (SFG). Like the second toroid, this small female glomerulus is included in the spheroidal array of glomeruli (black sphere S in Figures 2 X+ and 3 Y–). Its diameter is ~65 µm. If this interpretation is correct, then it follows that the MGC and the female glomerular complex each comprises three glomeruli: cumulus, first and second toroid in the male, two large (lLFG and mLFG) and one small (SFG) glomeruli in the female. This prompted the use of the term 'dimorphic' instead of 'specific' to refer to the two previously recognized glomeruli of the female glomerular complex.

Discussion

Based on the results reported here, the questions raised at the outset of this study can now be answered. (i) The three glomeruli composing the male MGC are not unique to males, but have homologs in females. (ii) The two large female glomeruli (LFGs) in the female are homologous to the two largest glomeruli (cumulus and first toroid) of the male MGC. The second toroid can be identified tentatively with a normal-sized glomerulus of the female, the small female glomerulus. (iii) No other conspicuous (qualitative) sexual dimorphism was demonstrated among the 'ordinary' glomeruli that make up the rest of the glomerular neuropil. These conclusions call for the following comments.

Absence of conspicuous sexual dimorphism among ordinary glomeruli

A similar absence of conspicuous sexual dimorphism among ordinary glomeruli has been reported in the cockroach *Blaberus craniifer* (Chambille and Rospars, 1985). It stands in contrast with the situation reported in the moth *Mamestra brassicae* (Rospars, 1983), where such dimorphisms were described, some of them consisting in the apparent subdivision of male glomeruli in the females, and others consisting in the displacement of several glomeruli in the vicinity of the MGC. Another form of sexual dimorphism, revealed by statistical analyses in both *Blaberus* and *Mamestra*, was the greater variability in locations and sizes of homologous glomeruli between individuals of opposite sexes than in individuals of the same sex (Rospars and Chambille, 1989). Examination of the computer reconstructions (Figures 2–4) suggests that this quantitative kind of dimorphism, indicative of fine structural variations, is also present in *Manduca*. It is not yet known, however, to what extent the intersexual variability exceeds the normal intrasexual variability in this species.

Homology of male and female macroglomerular complexes

According to the evidence presently at hand, it seems that the three glomeruli composing the male MGC can be identified with three glomeruli of the female. When all other glomeruli have been identified in males and females, only these three glomeruli remain. If one leaves aside the question of the number of glomeruli composing the glomerular complexes, then this interpretation in *Manduca* fits those given previously for *Blaberus* and *Mamestra*. *Blaberus* presents the clearest intersexual homology because it possesses a single sexually dimorphic glomerulus, which is twice as large (in volume) in the male as in the female and is actually the largest glomerulus in this species (Chambille and Rospars, 1985). Notably, the nymphal precursor of this macroglomerulus is not sexually dimorphic; the dimorphism appears only during the preimaginal phase, when the last nymphal stage transforms into the adult (Rospars and Chambille, 1986). In *Mamestra*, the male MGC comprises two large glomeruli located at the entrance of the antennal nerve into the AL. Two very small glomeruli are present in the same region in the female (Rospars, 1983). Nothing is known about the function of these female homologs, although it can be speculated that they also process sex-pheromonal information used by calling females for keeping optimal distance from other conspecific, calling females (Priesner, 1979; Ochieng' *et al.*, 1995; Schneider *et al.*, 1997).

The organization in *Bombyx*, however, suggests a different interpretation. In this species the male MGC consists of six glomeruli, two of which were considered as present in the female and the others as absent (Koontz and Schneider, 1987). The first two, which are enlarged in female *Bombyx*, can be interpreted as homologous to the large female glomeruli of *Manduca*, whereas the last four seem to be homologous to the cumulus and toroids of *Manduca*. If this interpretation is correct, then the MGC of *Bombyx* would be sex-specific and its LFGs, sexually dimorphic. Clarification of this issue will require further comparative investigations, with identification of the full complement of glomeruli.

On the basis of the relative prominence of the sexual dimorphism, *Manduca* would stand approximately midway between *Blaberus* and *Mamestra*. The large difference in shape, size and consequently location of the sexually dimorphic glomeruli make their homology less obvious in *Mamestra* and *Manduca* than in *Blaberus*. In the two lepidopteran species, to consider those glomeruli as homologous is simply the most economical hypothesis. Moreover, it is supported by developmental studies in *Manduca* showing that the three glomeruli of the MGC and the two LFGs are the first to form during the early stages of metamorphic adult development (Rössler *et al.*, 1998).

Nevertheless, these anatomical and developmental common features do not establish common functions.

The locations and forms of the glomeruli of the male MGC suggest that space constraints were determinant in the evolution of their structure. Their position at the entrance of the AL minimizes the length of ORC axons (Rospars, 1983). The toroidal shapes of part of their glomeruli leave room for neurites of AL neurons in the center of the complex, while keeping enough external surface for peripheral access of ORC axons. The non-toroidal shape of their female homologs suggests that space constraints are less stringent in the female.

Number of glomeruli

According to the present study, the canonical total number of glomeruli in both male and female *Manduca* is 63, with 60 ordinary glomeruli common to males and females and three sexually dimorphic glomeruli belonging to specialized glomerular complexes. This estimate differs from the previous estimate of 64 ± 1 ordinary glomeruli (Rospars and Hildebrand, 1992) for two reasons.

First, the structure of the male MGC has been revised. Three glomeruli (G1, G11 and G24) that were previously considered to be ordinary glomeruli now have been recognized as forming the second toroid (T2). The initial interpretation of this mass resulted from its horseshoe shape, narrower in its middle part, extending perpendicularly to the sectioning plane, and to its actual or apparent subdivision in two of the ALs that have been analysed. Whether this subdivision is an exceptional feature is not known.

Second, the anomalous status of one uncertain glomerulus has been confirmed. There was no doubt for the unilateral glomeruli found in one of the male brains (Msm1) and the female brain; those are clearly anomalous. However, the status of G38 observed bilaterally in one male brain (Msm1) but not in the other (Msm2) was uncertain. Now it appears that G38 cannot be found in the female brain. For this reason, it seems likely that this is a truly anomalous and supernumerary glomerulus that can be removed from the canonical count.

A comparative study taking into account more male and female ALs is in progress to test further and document quantitatively the main conclusion of the present report—that male and female ALs of *Manduca* have no sex-specific glomeruli, but only sexually dimorphic glomeruli, as far as anatomy is concerned. This conclusion has obvious physiological, developmental and evolutionary implications, which we hope to pursue in ongoing studies.

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