Male moths bearing transplanted female antennae express characteristically female behaviour and central neural activity

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SUMMARY

The primary olfactory centres of the sphinx moth *Manduca sexta*, the antennal lobes, contain a small number of sexually dimorphic glomeruli: the male-specific macroglomerular complex and the large female glomeruli. These glomeruli play important roles in sex-specific behaviours, such as the location of conspecific females and the selection of appropriate host plants for oviposition. The development of sexually dimorphic glomeruli depends strictly on the ingrowth of sex-specific olfactory receptor cell afferents. In the present study we tested the role of female-specific olfactory receptor cells (ORCs) in mediating female-specific host plant approach behaviour and in determining the response of downstream antennal lobe neurons. We generated male gynandromorphs by excising one imaginal disc from a male larva and replacing it with the antennal imaginal disc from a female donor. Most male gynandromorphs had an apparently normal female antenna and a feminised antennal lobe. These gynandromorphs were tested for flight responses in a wind tunnel towards tomato plants, a preferred host plant for oviposition in *M. sexta*. Male gynandromorphs landed on host plants as often as normal females, demonstrating that the presence of the induced female-specific glomeruli was necessary and sufficient to produce female-like, odour-oriented behaviour, i.e. orientation towards host plants. We also characterised the physiological and morphological properties of antennal lobe neurons of male gynandromorphs. We found that projection neurons with arborisations in the induced female-specific glomeruli showed physiological responses akin to those of female-specific projection neurons in normal females. These results therefore indicate that ORCs confer specific odour tuning to their glomerular targets and, furthermore, instruct odour-specific behaviour.

Key words: *Manduca sexta*, development, olfaction, gynandromorph, behaviour.

INTRODUCTION

Olfactory-mediated host plant recognition by herbivorous insects depends not only on the characteristics of a plant but also on the odour-detection capabilities of the herbivore's olfactory system (Hanson and Dethier, 1973). The antennae, the main olfactory organ of insects, carry thousands of hair-like, multiporous olfactory sensilla, each containing one to a few olfactory receptor cells (ORCs). The axons of ORCs project *via* the antennal nerve to the antennal lobe (AL), the primary olfactory centre of insects.

Each adult antenna develops from an imaginal disc that everts at the onset of adult metamorphosis (Sanes and Hildebrand, 1976). ORCs send axons to the developing AL where they induce the development of dense areas of neuropil called glomeruli (Oland and Tolbert, 1987; Oland and Tolbert, 1988; Oland et al., 1988). Glomeruli are uniquely identifiable, functional units of the AL in vertebrates and invertebrates alike and the first synaptic sites within the olfactory system (Hildebrand and Shepherd, 1997). Glomeruli are composed of the densely packed neurites of, and of synapses among, the afferent ORCs, AL neurons (local interneurons and output projection neurons) and centrifugal neurons, surrounded by a sheath of glial cells (Tolbert and Hildebrand, 1981). Glomeruli are units dedicated to synaptic processing of primary-afferent information about a particular odour compound, related compounds or certain molecular attribute(s) of the odour compound(s) (reviewed in Buck, 1996; Hildebrand and Shepherd, 1997; Ache and Young, 2005; Mori et al., 2006).

As in many moths, the antennae of the moth *Manduca sexta* L. (Lepidoptera: Sphingidae) are sexually dimorphic. Female antennae possess only short-to-medium-length sensilla whereas the antennae of males carry additional male-specific type-I long trichoid sensilla (Kaissling et al., 1989). The sexual dimorphism of the antenna is reflected in the presence of sexually dimorphic glomeruli in the ALs, the male-specific macroglomerular complex (MGC) and the large female glomeruli (LFG) (Rospars and Hildebrand, 1992; Rospars and Hildebrand, 2000). Male-specific ORCs and AL neurons respond sensitively and selectively to the two main components of the female sex pheromone (Kaissling et al., 1989; Christensen and Hildebrand, 1987; Hansson et al., 1991; Heinbockel et al., 1999). Likewise, because they are present only in females and contain projection neurons (PNs) that respond to host plant volatiles, the LFGs are thought to mediate female-specific behaviours, such as the location and/or selection of appropriate host plants for oviposition (King et al., 2000; Reisenman et al., 2009).

The role of ORCs in the formation of sexually dimorphic glomeruli was demonstrated by trans-sexual transplantation experiments conducted at the larval stage; ORCs from a grafted male antenna induced the formation of an MGC in a host female whereas ORCs from a grafted female antenna induced the formation of LFGs in a host male (Schneiderman et al., 1982; Rössler et al., 1999). Adult females developed an apparently normal male antenna whose axons innervated a masculinised AL with neurons that responded to stimulation with the female sex pheromone (Schneiderman et al., 1982). Moreover, these gynandromorph females responded to pheromonal stimulation with a casting behaviour typical of males (Schneiderman et al., 1986). These earlier findings prompted us to perform the reciprocal experiment. We

sought to test if transplantation of a female antennal imaginal disc to the head of a male larva, in place of its own disc, would not only feminise the male AL but also feminise the odour-modulated behaviour of the gynandromorph moth, just as was shown in the case of female gynandromorph expressing male-specific behaviour in response to sex pheromone. We seek to: (a) discover if the ability of sex-specific olfactory axons to induce or permit correspondingly sex-specific AL development is a general phenomenon or one that is strictly male-specific; (b) test if such altered central nervous system development consistently leads to corresponding behavioural consequences; (c) add evidence to a growing argument that male- and female-specific antennal ORCs feed (respectively, through male- and female-specific AL glomeruli) into neural circuitry underlying a common motor program controlling counterturning anemotactic flight; and (d) lay phenomenological groundwork for future efforts to discover the molecular mechanisms responsible for primary-afferent influences over AL development.

Female *M. sexta* use olfactory information to locate host plants for oviposition (Yamamoto et al., 1969; Sparks, 1969; Mechaber et al., 2002). The LFGs are female-specific glomeruli that process olfactory information about a limited range of odour molecules emitted by host plants (King et al., 2000; Reisenman et al., 2004). For example, projection neurons arborising in the lateral LFG (latLFG) show preferential responses to the common host plant volatile (±)-linalool, and particularly to the (+) enantiomer of that odour compound (King et al., 2000; Reisenman et al., 2004).

In the present study we tested the role of female ORCs in mediating female-specific host approach behaviour and in determining the response of downstream AL olfactory neurons. We generated antennal gynandromorphs (insects with an antenna of the opposite sex) by transplanting the antennal imaginal disc of larva to a larva of the opposite sex. In wind tunnel experiments, we studied the host plant approach behaviour of normal males and females, male gynandromorphs (males with one female antenna and a feminised AL), female gynandromorphs (females with one male antenna and a masculinised AL) and that of unilaterally antennectomised male and female moths (i.e. insects with only one antenna and one AL). We furthermore investigated the physiological consequences of ORC-induced feminisation of the AL, by studying the morphological and physiological properties of AL neurons in male gynandromorphs.

MATERIALS AND METHODS
Animals
Manduca sexta were reared from eggs on artificial diet under a long-day photoperiod [17 h:7 h light:dark (L:D) cycle] at 26°C and 50–60% relative humidity. Male and female pupae used in behavioural experiments were removed approximately 15 days prior to adult emergence, segregated by sex and maintained on a reversed 14 h:10 h L:D cycle at 26°C:24°C (L:D). Two days before adult eclosion pupae were transferred to fibreglass-screen emergence cages (31 cm × 31 cm × 32 cm), still segregated by sex. At no time prior to behavioural experiments were moths exposed to plant odours. Moths were tested in behavioural bioassays 1.5–3 h after the onset of the scotophase.

Antennal transplants
Antennal gynandromorphs of both sexes were produced following the surgical technique developed by Schneiderman et al. (Schneiderman et al., 1982). Antennal imaginal discs were transplanted unilaterally on the 2nd–3rd day of the fifth (final) larval instar. For this, larvae were restrained in aluminium tubes and anaesthetised by cooling on ice for at least 30 min. The head cuticle was cut with a microscalpel around the base of one antenna to remove the larval antenna and the underlying antennal imaginal disc, and a mixture of antibiotics (phenylthiourea, streptomycin sulphate and penicillin, 2:1:1) was applied to the wound. The corresponding antenna and imaginal disc from a donor of the opposite sex was implanted and sealed with melted myristic acid. Larvae were removed from the tubes, dried and placed in clean cups with food. Unilaterally antennectomised moths of both sexes were produced by unilateral removal of one antennal imaginal disc. After pupation these animals lacked an antenna on the operated side or developed a stunted antenna with no connection to the brain but otherwise appeared normal. As in the transplantation experiments the opposite side remained intact.

Histology
Brains of gynandromorphs and antennectomised moths were excised, desheathed and viewed under a binocular dissecting microscope (Wild, Heerbrugg, Switzerland). Thus, moths tested in behavioural experiments could be classified as belonging to one of four groups with respect to appearance and innervation of the antenna in the operated side. (1) Moths with no antenna and AN in the antennectomised side: these moths did not develop an antenna or an antennal nerve in the operated side. (2) Moths with a partial antenna and no antennal nerve in the antennectomised side: these moths developed an antenna that was shorter than the normal length of the same sex but lacked an antennal nerve. (3) Gynandromorphs with a partial antenna of the opposite sex but no antennal nerve in the transplanted side: these moths developed a shortened antenna with characteristics of the donor sex but lacked an antennal nerve on the operated side. (4) Gynandromorphs with a complete antenna and AN of the opposite sex in the transplanted side: these moths developed a full-sized antenna of the opposite sex with an antennal nerve substantially innervating the ipsilateral AL. Brains were fixed overnight in 2% glutaraldehyde in 0.1% phosphate buffer, dehydrated by passage through a graded series of ethanol solutions and cleared in methyl salicylate. Brains were embedded in Spurr's resin (Electron Microscopy Sciences, Ft Washington, PA, USA), sectioned at 48 μm using a sliding microtome, and mounted and imaged (2 or 5 μm optical sections) with a laser-scanning confocal microscope (MRC-600; Bio-Rad, Cambridge, MA, USA, equipped with a Krypton/Argon and an Argon laser).

Behavioural experiments
Behavioural responses of three-day-old unmated moths were observed in a Plexiglas wind tunnel (4 m long × 1.2 m wide × 1.2 m tall). Charcoal-filtered air was forced through the upwind end of the wind tunnel (velocity=0.5 m s^{-1}) using nine axial fans (0.25 m diameter). An almost laminar air flow was created by forcing air passage through a cheesecloth, a shade cloth and an organdy fabric screen. Odour-laden air was exhausted at the downwind end through an aluminium scoop (65 cm width × 103 cm height) connected to an exhaust fan. The floor of the wind tunnel had a visual pattern consisting of randomly arranged 10 cm-diameter red circles on a white background that provided visual feedback. Illumination was provided with red incandescent bulbs (*ca*. 2 lx). Odour stimuli originated from a potted tomato plant (*Lycopersicon esculentum* Miller, variety Money Maker, Champion or Early Girl) placed 20 cm from the upwind end of the wind tunnel. Plants were grown in a greenhouse from seed and transferred from the greenhouse to the wind tunnel immediately before testing. The flight trajectories of moths were recorded with two low-light video cameras (Model

TC351A, Burle Inc., Ireland, equipped with a Javelin lens model JL 12575, Ademco, Torrence, CA, USA).

Moths were placed individually in $14\,cm \times 30\,cm$ cylindrical screen cages and allowed to acclimate to room conditions for 30–60 min before testing. Moths were introduced singly to the downwind end of the wind tunnel by placing the screened cage on a 0.3 m-tall (plant foliage height) release platform. We estimated the location and dimension of a typical odour plume from the tomato odour source by releasing titanium tetrachloride smoke at the plant foliage place and height. Moths usually started wing fanning within 30 s; those that remained quiescent were stimulated to initiate wing fanning by moving the cage in and out of the odour plume (Willis et al., 1995). A flight was deemed a positive response if the moth showed odour-guided, zig-zagging upwind flight in the wind tunnel and landed on the tomato plant.

Logistic regression models were performed to compare the landing behaviour of moths in the different experimental groups. In these models the combined factors 'group and sex' and 'tomato variety' were included in order to study the influence of sex and plant varieties. Results were considered significant when $P<0.05$. Odds ratios and 95% confidence intervals were calculated from the corresponding models. All analyses were done using appropriate software [R version 2.7.0. (R Development Core Team, 2005)].

To test if *M. sexta* use visual cues to locate host plants, a three-dimensional green tomato plant model made from paper colour copies of a real tomato plant was used. The stem of the model was strengthened with wire, which was covered with green paper. This plant model, with a shape and size similar to that of tomato plants, was placed in the wind tunnel in the same position as real plants. Flight trials with the paper model were performed as described above.

Electrophysiological experiments

Gynandromorph males were used 1–3 days after adult emergence. Animals were dissected and prepared for intracellular recording according to established procedures (Reisenman et al., 2004). After mechanical removal of the perineural sheath covering the AL, the preparation was continuously superfused with physiological saline solution containing (in mmol l^{-1}): 150 NaCl, 3 CaCl$_2$, 3 KCl, 10 TES buffer (pH 6.9) and 25 sucrose (Christensen and Hildebrand, 1987). For odour stimulation, the cut end of the grafted antenna was inserted into a glass capillary tube filled with physiological saline solution, which served both as a holder to suspend the antenna in the air in front of the odour-delivery glass tube, and as an electrode for monitoring antennal responses to olfactory stimulation. An L-shaped glass tube delivered a constant flow of humidified, charcoal-filtered air to the antenna (1.9 l min^{-1}). Odour compounds were injected (2 ml volume, 200 ms pulse duration) into the constant air stream *via* a computer-driven syringe olfactometer (Reisenman et al., 2004). A funnel connected to a negative-pressure line was positioned near and behind the preparation to remove stimulus volatiles after delivery to the antenna. The odour compounds used were: (–)-linalool [(–)3,7-dimethyl-1,6-octadien-3-ol, catalogue no. 62139, >95%; Fluka, Buchs, Switzerland], (+)-linalool [(+)3,7-dimethyl-1,6-octadien-3-ol, 99.9% pure based on gas chromatographic analysis; obtained as described in Reisenman et al. (Reisenman et al., 2004)] and *cis*-3-hexenyl-acetate (catalogue no. H2137, >97% pure; Tokyo Chemical Industries, Tokyo, Japan). (±)-Linalool and *cis*-3-hexenyl-acetate are found among the volatiles emitted by host plants of *M. sexta*, including tomato (Fraser et al., 2003; Lougrhrin et al., 1990; Raguso et al., 2003). We used (+)- and (–)-linalool because we previously shown that female-

specific neurons in normal females discriminate between the enantiomers of linalool (Reisenman et al., 2004). Therefore, the use of these odour compounds provides a very powerful tool to test whether female antennal transplants induced the development of female-specific glomeruli containing neurons with odour-response characteristics akin to those of normal females. Moreover, linalool participates in mediating oviposition behaviour in *M. sexta*, with (+)-linalool and (–)-linalool, respectively, producing attraction and repellence for oviposition in normal females (Reisenman et al., 2010). *Cis*-3-hexenyl-acetate was used because we have shown that this odorant activates projection neurons in a sexually isomorphic glomerulus that neighbours the LFGs, which has similar effects in both male and female neurons (Reisenman et al., 2005). Single odour compounds were diluted to a concentration of 1:500 in odourless mineral oil (Sigma-Aldrich, St Louis, MO, USA); 50 µl of solution was applied to a disc of filter paper and inserted into a 20 ml stimulus syringe (Reisenman et al., 2004). Control cartridges contained a piece of filter paper loaded with 50 µl of solvent (mineral oil).

Sharp microelectrodes were made from borosilicate glass capillaries with filament (1 mm o.d., 0.58 or 0.75 mm i.d., Sutter Instruments Co., Novato, CA, USA) on a laser puller (P-2000, Sutter Instruments Co.). The tip of the electrode was filled with a 65 mmol l^{-1} solution of Lucifer Yellow CH (Sigma-Aldrich) in 200 mmol l^{-1} LiCl or with a solution of Alexa Fluor 568 hydrazide (10 mmol l^{-1} in 200 mmol l^{-1} KCl, Molecular Probes, Eugene, OR, USA), and the shaft was filled with 2 mol l^{-1} LiCl. The responses of an impaled neuron to stimulation of the ipsilateral antenna were amplified 10-fold and digitised at 20 kHz (Axoclamp-2A, Digidata 1200 series Interface, Axon Instruments, Foster City, CA, USA). After electrophysiological recordings were obtained, neurons were injected with either Lucifer Yellow or Alexa 568 (see above) by passing hyperpolarising current (0.2–1 nA) for 6–40 min. Upon completion of an experiment, the brain was excised and immersed in 2.5% formaldehyde fixative solution, dehydrated and cleared as described above. Cleared brains were imaged as whole mounts (optical sections, 2 µm thick) with a laser-scanning confocal microscope.

RESULTS

Gynandromorph males developed an apparently normal adult female antenna on the transplanted side, i.e. without the long type-I trichoid sensilla characteristic of normal males (Fig. 1). Similarly, gynandromorph females developed an antenna with male characteristic long type-I trichoid hairs at the transplanted side. Thus, as shown by Schneiderman et al. (Schneiderman et al., 1982), we found that all transplanted antennae exhibited a morphology characteristic of the donor's sex.

Antennal nerves, ALs and optic lobes were clearly visible in dissected brains of normal male and female moths (Fig. 2A). In cases in which one imaginal disc was removed at the larval stage and not replaced, no antennal nerve developed and the AL was stunted (Fig. 2B, referred to as moths with no antenna and AN in the antennectomised side). In some cases surgical removal of the imaginal disc was incomplete and a stunted antenna (2–30% of normal length) with morphological characteristics of the individual's sex developed but did not form an antennal nerve (referred to as moths with a partial antenna of the same sex and no antennal nerve in the antennectomised side). Gynandromorph brains exhibited ingrowing axons from antennal transplants of the opposite sex and an antennal nerve innervating the AL in the transplanted side (Fig. 2C, referred to as gynandromorphs with an antenna of the
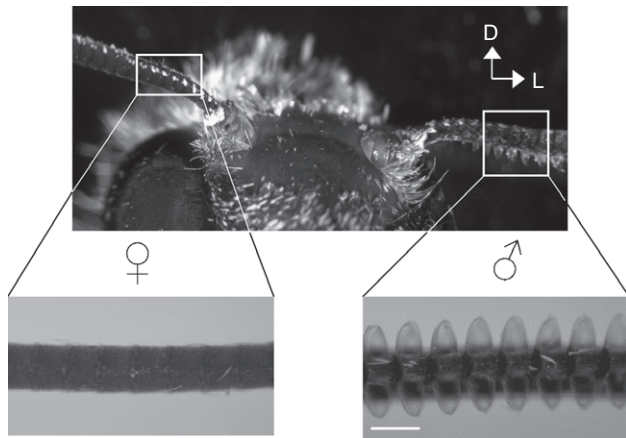
Fig. 1. Gynandromorph male *Manduca sexta* with a normal male antenna (right) showing the long hair-like trichoid sensilla and a characteristic female antenna on the left without trichoid sensilla. D: dorsal; L: lateral. Scale bar: 500 μm. Inserts: detail of the male and the female (donor) antenna.
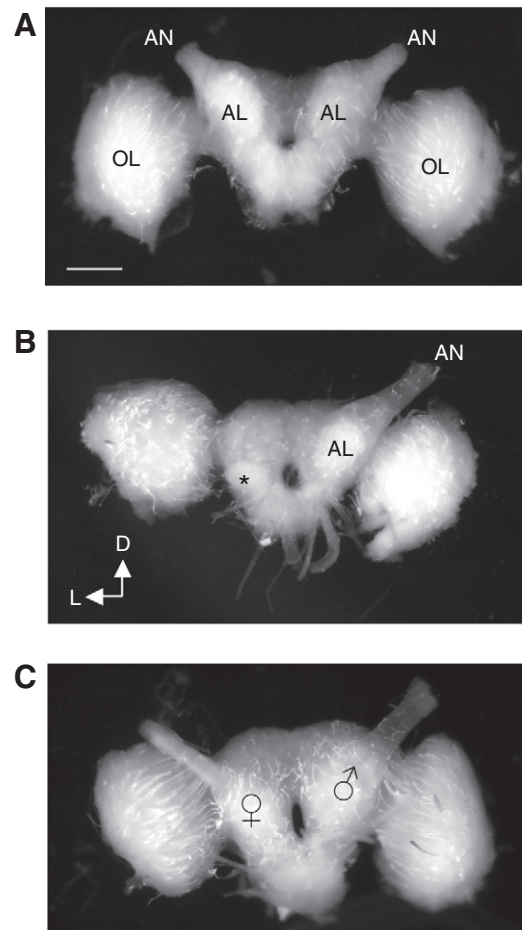

Fig. 2. (A) Brain of a normal moth showing the antennal nerves (AN), antennal lobes (AL) and optic lobes (OL). D: dorsal, L: lateral. Scale bar: 350 μm. (B) Brain of a male moth with no antennal nerve and a stunted AL (*) in the operated side (moths missing one antenna and AN); the imaginal disc was removed in the last larval instar. (C) Brain of a male gynandromorph with a transplanted female antenna that successfully developed both an AN and an AL.

opposite sex and an antennal nerve in the transplanted side). Some gynandromorphs developed a shorter antenna (5–95% of normal length) with morphological characteristics of the donor's sex but lacked an antennal nerve (referred to as gynandromorphs with a partial antenna of the opposite sex but no antennal nerve in the transplanted side).

Histological sections from the brains of normal males, male and female antennal gynandromorphs, and moths that had one antenna surgically removed at the larval stage (antennectomised moths) were examined. Normal males showed an MGC at the entrance of the antennal nerve into the AL (Fig. 3A). In male moths where one antenna was removed at the larval stage, the AL on the operated side was stunted and did not receive antennal innervation (Fig. 3B, left); the AL on the intact side (control side, right) was innervated by the antennal nerve and showed a male-characteristic glomerular organisation. Male gynandromorphs with an antenna and an AN of the donor sex developed the characteristic female-specific LFG on the AL innervated by the female graft (Fig. 3C, left) and a characteristic male-specific MGC on the intact side (Fig. 3C, control side, right). Similarly, female gynandromorphs developed the MGC characteristic of males in the AL innervated by the male graft (Fig. 3D, left) and the typical female LFG on the intact side (Fig. 3D, control side, right).

Behavioural experiments

Only 8% and 10% of normal males and females, respectively, landed on the tomato paper model (Fig. 4A), indicating that visual cues alone are not sufficient to guide host-seeking behaviour. By contrast, normal females landed on the tomato host plant more often than normal males [97% *vs* 40%, P=0.0005, odds ratio (OR) and confidence intervals=43.4 (5.2, 363), N=30 per sex, Fig. 4B].

Among unilaterally antennectomised moths [i.e. neither antenna nor antennal nerve (AN) developed], 32% of females (N=28) and 33% of males (N=39) landed on host plants (Fig. 4C). Significantly fewer of these unilaterally antennectomised females landed on tomato plants compared with normal females [P=0.0001, OR=65.1 (7.6, 558), N=58]. In cases in which one antennal imaginal disc was removed but nevertheless a stunted antenna – but no antennal nerve – developed (females: 3–30% of normal length, mean=9.4%; males:

2–20% of normal length, mean=7.4%), 62% of females (N=13) and 27% of males (N=15) landed on the host plant (Fig. 4D). The differences in landing between females with a partial antenna of the same sex and no AN, and females lacking one antenna (and the respective AN) were not significant (P=0.10, N=41). Among gynandromorphs with a partial antenna of the opposite sex but no AN (females: 5–90% of normal length, mean=58%; males: 10–95% of normal length, mean=59%), 63% of females (N=8) and 40% of males (N=10) landed on the host plant (Fig. 4E). All female gynandromorphs that developed a complete male antenna, an AN and an AL with male characteristics (N=4) landed on the host plant. Although a low number of moths were tested, these results indicate that the presence of just one female antenna and the respective female-specific AL is sufficient to mediate olfactory-mediated host-plant approach. Among those male gynandromorphs that developed a complete female antenna, an AN and a feminised AL, 88% landed on host plants (N=16; Fig. 4F). These male gynandromorphs landed on host plants significantly more often than did normal males [P=0.007, OR=0.1 (0.02, 54), N=46] and gynandromorph males that developed a stunted female antenna but
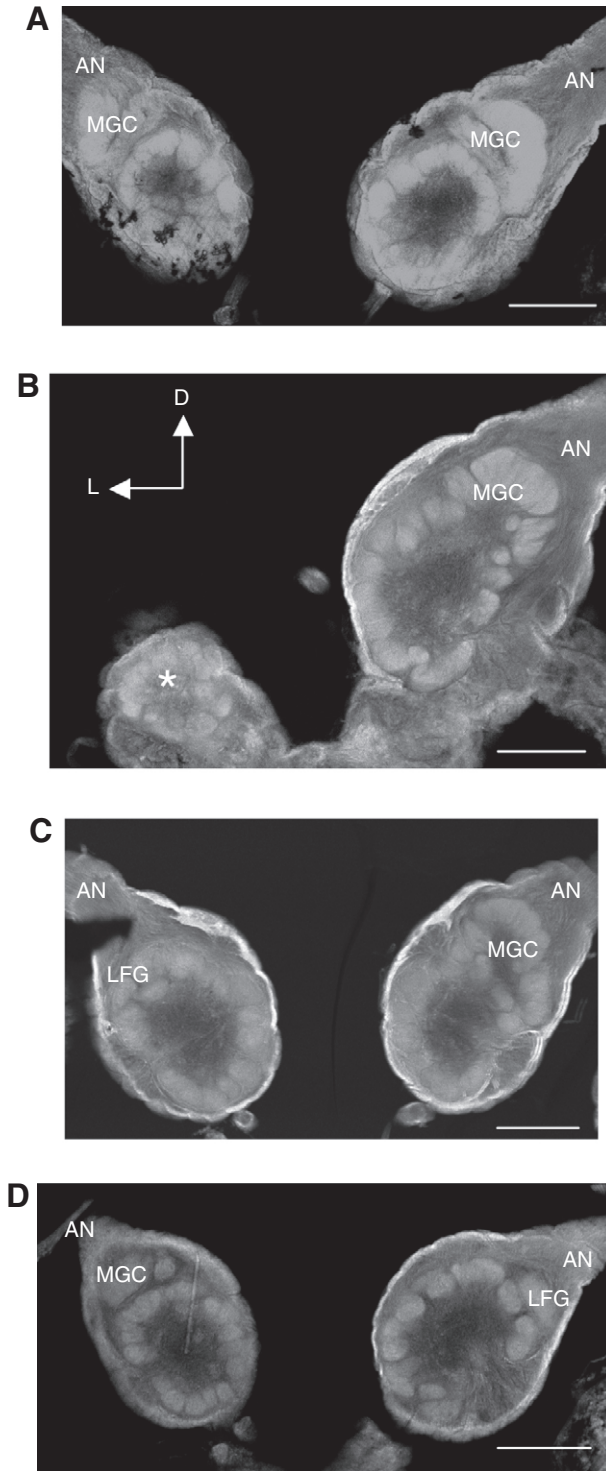
Fig. 3. (A) Frontal section through the antennal lobe (AL) of a normal male showing both antennal nerves and the characteristic male macroglomerular complex (MGC) on both sides. (B) Male moth with one antenna missing. The antennal nerve (AN) is missing and the AL stunted (*) in the side where the antennal imaginal disc was removed. (C) Male gynandromorph with an antenna of the opposite sex (with an AN) shows a typical male MGC structure on the control (un-operated) side and the induced large female glomeruli (LFGs) typical of female moths on the transplanted side. (D) Female gynandromorph with an antenna (and AN) of the opposite sex shows the LFGs on the control (un-operated) side (only one of the LFG is visible in this section) and the male characteristic MGC on the transplanted side. Scale bars: 200 μm. D: dorsal; L: lateral.
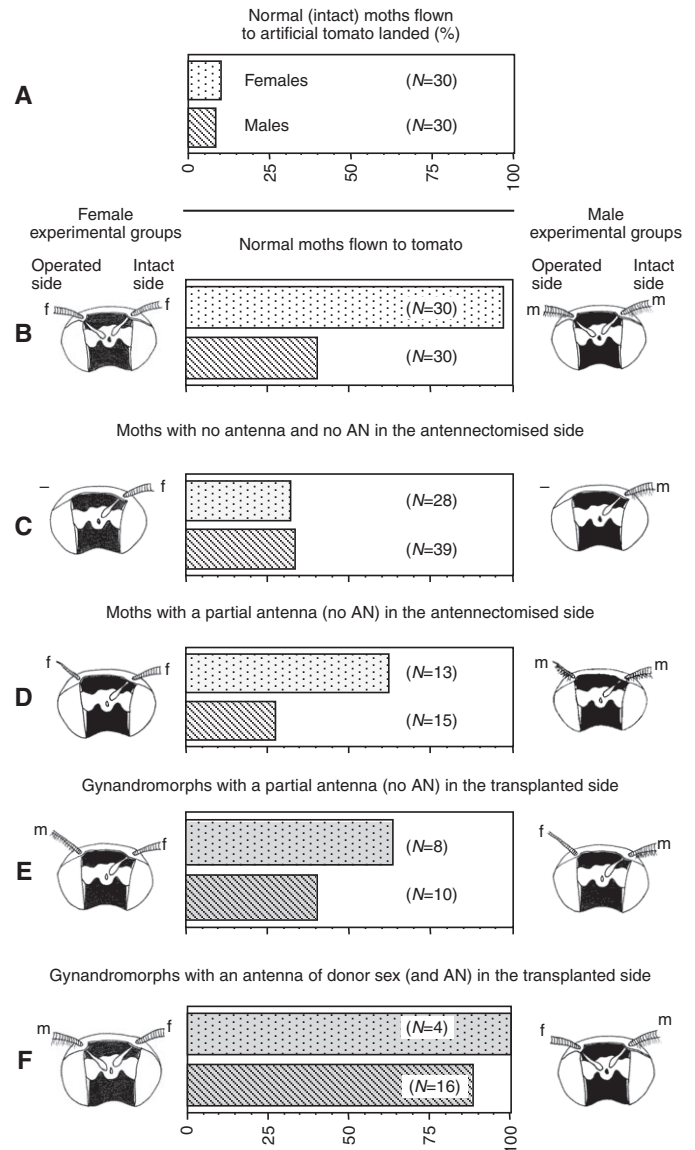

Fig. 4. Flight responses (% landed) of normal (A,B), antennectomised (C,D) and gynandromorph (E,F) females (dotted bars) and males (line bars) to tomato plants. Normal (intact) moths were also flown to an artificial (odourless) tomato plant (A). The brain of each moth was dissected after behavioral testing to establish whether the removed or transplanted antenna formed an antennal nerve (AN) innervating the antennal lobe (AL); animals were then assigned to each group (cartoons, B–F). Numbers between parentheses indicate the number of animals tested in each group. (B): Flight response of normal (intact) moths to a potted tomato host plant; (C): flight responses of moths with no antenna and AN in the antennectomised side; (D): flight responses of moths with a partial antenna but no AN in the antennectomised side; (E): gynandromorphs with a partial antenna of the opposite sex but no AN in the transplanted side; (F): gynandromorphs with an antenna of the opposite sex and an AN in the transplanted side. m, male; f, female.

no AN [$P=0.017$, OR=0.09 (0.01, 66), $N=26$]. Furthermore, they landed on host plants as often as normal females ($P=0.25$, $N=46$; Fig. 4B,F).

The different varieties of tomato used in these experiments did not significantly affect the behaviour of moths ($P>0.2$).

Normal females and gynandromorph males with an AN exhibited similar flight tracks in the wind tunnel. They first circled around
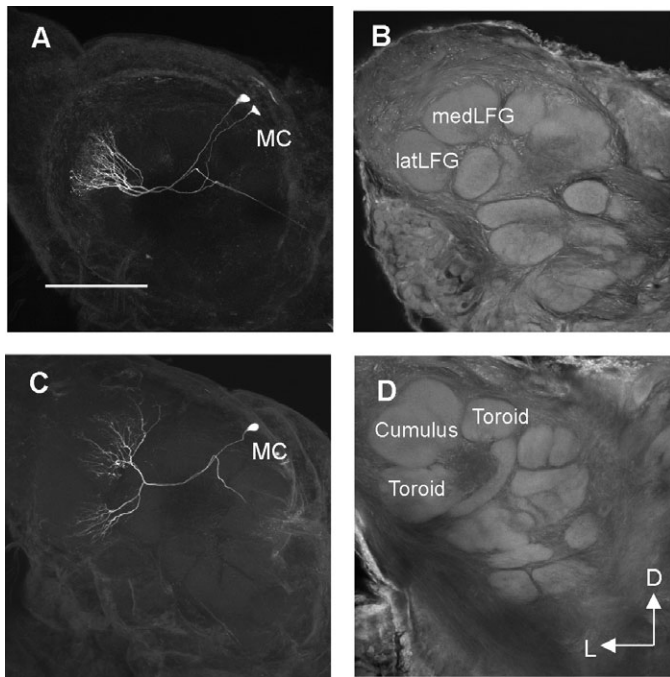
Fig. 5. Laser-scanning confocal microscopic images of two female-specific projection neurons with arborisations confined to the lateral female glomeruli (latLFG) in a normal female (A) and of a male-specific projection neuron with arborisations restricted to the Toroid I glomerulus of the MGC in a normal male (C). The cell bodies of sex-specific neurons reside in the medial cluster of neuronal cell bodies (MC). Confocal microscopic images of glutaraldehyde-intensified normal female (B) and male (D) ALs showing, respectively, the positions of the female-specific LFGs (the latLFG and the medial LFG, medLFG) and the male-specific MGC. Scale bar: 200 μm. D: dorsal; L: lateral.
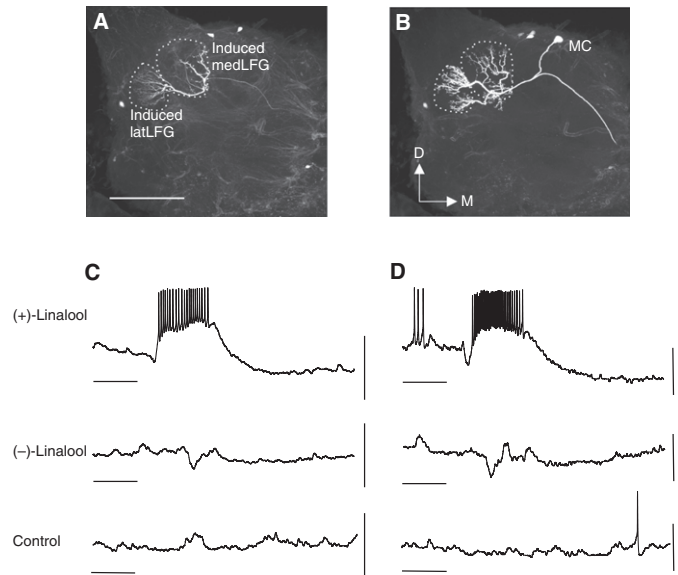

Fig. 6. (A,B) Laser-scanning confocal microscopic images of projection neurons from a gynandromorph male with arborisations restricted to the induced lateral female glomeruli (latLFG) (dotted lines). These two projection neurons were stained with different dyes in the same animal. Scale bar: 200 μm. D: dorsal; M: medial. Note that as in normal animals, the cell bodies of these neurons are in the medial cluster (MC) of neuronal cell bodies. These neurons arborise in the two induced LFGs. (C,D) Electrophysiological responses obtained from the projection neurons shown in A,B to stimulation with (+)-linalool, (–)-linalool and the mineral oil control (linalool was tested at 1:500 vol./vol.). The activation of the odour-delivery stimulation system is indicated in the bars below records (duration=200 ms). Calibration bars are 5 mV (C) and 1 mV (D).

the release cage before following the odour plume, approached the host in a characteristic zig-zag path that narrowed as they drew close and finally landed on the tomato host plant.

In sum, these results show that female ORCs induce the development of female-specific olfactory structures and that gynandromorph males with such a feminised AL exhibit female-like odour-oriented behaviour, i.e. animals fly towards odours that typically evoke responses in normal females but not in normal males.

Electrophysiological experiments

Having found that a female graft induces the formation of a feminised AL and behavioural responses towards host plant odours, which typically attract females (Figs 3 and 4), we asked whether AL neurons in male gynandromorphs show female-specific odour responses. In normal females, AL projection neurons in the two LFGs respond to host plant volatiles (King et al., 2000; Reisenman et al., 2004; Reisenman et al., 2010). Projection neurons in the latLFG, an example is shown in Fig. 5A,B) respond preferentially to antennal stimulation with (±)-linalool (King et al., 2000) and, particularly, to the (+) enantiomer of this odour compound (Reisenman et al., 2004). For comparison, an example of a projection neuron from an un-operated (intact) male with arborisations restricted to the toroid MGC glomerulus is shown in Fig. 5C,D.

Fig. 6A,B show two examples of female-specific projection neurons from a male gynandromorph with arborisations restricted to the induced LFGs. As in normal moths (Fig. 5A,C), the cell body of these sex-specific projection neurons are located in the medial cluster of neuronal cell bodies. Different from LFG projection neurons of normal females, the projection neurons shown in this example have arborisations in the two LFGs (compare Fig. 5A and Fig. 6A,B). Antennal stimulation with (+)-linalool evoked a triphasic response in these neurons consisting of a brief inhibitory potential, followed by an excitatory phase with spiking and a delayed inhibitory phase characterised by membrane hyperpolarisation and suppression of spiking. This triphasic response is characteristic of some MGC-projection neurons in males and some projection neurons in sexually isomorphic glomeruli, i.e. glomeruli present in both sexes (e.g. Heinbockel et al., 1999; Guerenstein et al., 2004; Reisenman et al., 2005). Stimulation with (–)-linalool, by contrast, only evoked a hyperpolarisation (Fig. 6C,D). Thus, these projection neurons discriminate between the enantiomeric forms of linalool, as do latLFG-projection neurons in normal females (Reisenman et al., 2004).

Fig. 7 shows a population analysis of the responses of projection neurons recorded from male gynandromorphs to stimulation with the two enantiomers of linalool and the control stimulus. As a group, we found that projection neurons from male gynandromorphs (N=6, projection neurons recorded from four individuals; not all projection neurons could be completely stained) show a preferential response to the (+) enantiomer of linalool.

As in normal females (Reisenman et al., 2004), not all projection neurons in sexually isomorphic glomeruli in male gynandromorphs responded to stimulation with linalool or showed enantioselective responses. Fig. 8A shows one example of such a projection neuron
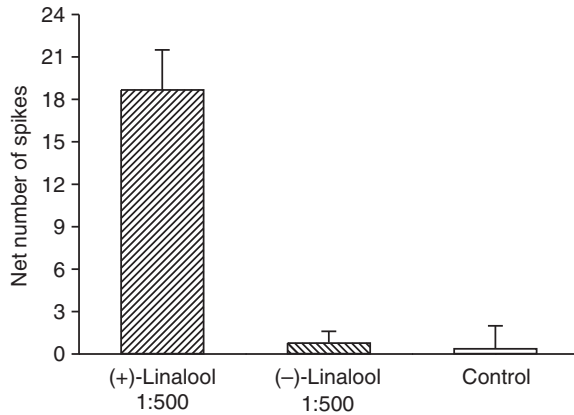
Fig. 7. Responses of projection neurons (net number of spikes, means ± s.e.m.) from male gynandromorphs (grouped data). Responses to (+)-linalool were statistically different from responses to (−)-linalool (Wilcoxon matched pairs test, $P<0.05$, $N=6$ projection neurons recorded from four gynandromorphs). Not all projection neurons could be morphologically identified. Linalool was tested at 1:500 vol./vol. (duration=200 ms).
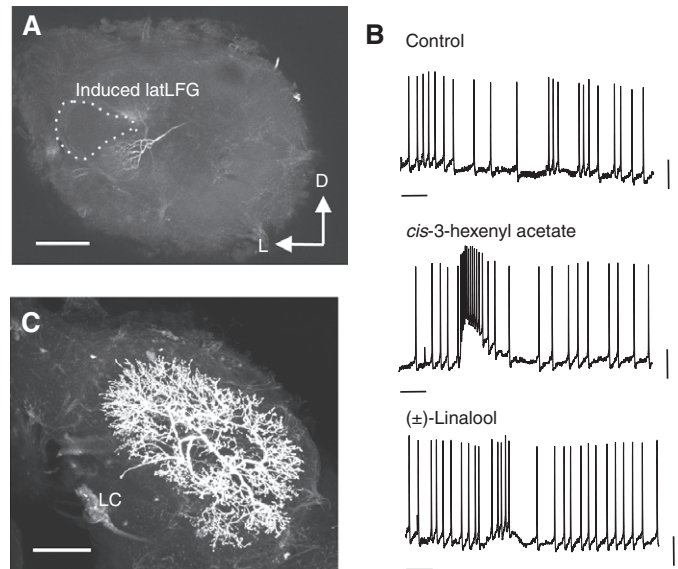

Fig. 8. (A) Microscopic confocal image of a projection neuron from a male gynandromorph with arborisations in a glomerulus neighbouring the induced lateral female glomeruli (latLFG) (dotted lines). Scale bar: 100 μm. (B) Physiological responses of the projection neuron in A to stimulation with cis-3-hexenyl acetate (1:10,000 vol./vol.) and (±)-linalool (1:100 vol./vol.). The activation of the odour-delivery stimulation system is indicated in the bars below records (duration=200 ms). Calibration bar: 2.5 mV. (C) Microscopic confocal image of a local neuron from male gynandromorphs; as in normal animals local neurons in male gynandromorphs have their cell body in the lateral cluster of neuronal cell bodies (LC). Scale bar: 100 μm. D: dorsal, L: lateral. The dotted lines indicate the outline of the antennal lobe.

with arborisations restricted to a sexually isomorphic glomerulus adjacent to the induced latLFG. This projection neuron shows a strong response to stimulation with low concentrations of the plant volatile cis-3-hexenyl-acetate and only a very weak excitatory response to stimulation with high concentrations of (±)-linalool (Fig. 8B). An example of a local interneuron is shown in Fig. 8C. This neuron has a morphology similar to that of normal moths (Reisenman et al., 2008).

In sum, these results show that projection neurons with arborisations in the induced LFGs have physiological characteristics similar to those of normal latLFG-projection neurons. Projection neurons in sexually isomorphic glomeruli, as expected, have physiological responses akin to those of normal animals.

DISCUSSION

We used an established surgical transplantation technique to generate antennal male and female gynandromorphs, i.e. animals with one antenna of the opposite sex (Schneiderman et al., 1982; Rössler et al., 1999). The donor antenna induced the formation of female- and male-specific glomeruli in the AL of male and female recipients, respectively. In behavioural experiments, male gynandromorphs with a feminised antenna and AL landed on host plants significantly more often than did normal males and as often as did normal females. These gynandromorphs landed on host plants more often than male gynandromorphs that developed a female antenna but no AN. Thus, the presence of a female-like AL was necessary to evoke olfactory-mediated orientation towards host plants in male gynandromorphs. We found that projection neurons with arborisations in the induced female-specific LFGs showed physiological responses akin to those of some LFG-projection neurons in normal females. Thus, these results show that ORCs confer specific odour tuning to their glomerular targets and instruct odour-specific behaviour. Importantly, our behavioural observations were unbiased, because animals could only be assigned to experimental groups after behavioural testing, when histological work was conducted.

As our surgical procedure alters and/or reduces the number of olfactory afferents, we first tested if visual cues alone could mediate host plant seeking behaviour. We found that about 10% of normal (un-operated) males and females landed on odourless tomato paper

models, indicating that moths do not land on the only present object if plant odours are missing. By contrast, more than 95% of normal females landed on real tomato plants. These results indicate that tomato vegetation provided an attractive olfactory cue that mediates female attraction to host plants for oviposition because (1) tomato flowers do not produce nectar and thus are not used as a food resource by adult moths, and (2) less than half of normal males landed on the tomato plant.

We next tested the consequences of unilateral antennectomisation (removal of one antenna during the larval stage) on the host plant seeking behaviour of adults. As previously reported (Hildebrand et al., 1979; Tolbert et al., 1983; Schweitzer et al., 1976), such animals did not form an AN in the operated side and developed a stunted AL with a few clearly abnormal glomerulus-like structures. These unilaterally antennectomised females landed on host plants significantly less often than normal females. This result suggests that bilateral input and/or glomerular organisation is necessary to fully evoke a typical female-like host-searching behaviour. We also found that females that developed a stunted antenna (but no antennal nerve) landed on tomato plants slightly more often (but the differences were not statistically significant) than females that did not develop an antenna at all at the operated side (compare Fig. 4C,D). We hypothesise that the stunted antenna might stabilise flight trajectories; thus, helping moths to locate the odour source with their intact antenna.

Successfully operated male gynandromorphs developed a female antenna lacking the typical male specific type-I trichoid sensilla but possessed an AN and an AL with the female-characteristic LFGs

on the operated side. These animals landed on the host plant as often as normal females and twice as much as normal males. Thus, the female graft not only induced the development of female-specific olfactory structures in the AL (Schneiderman et al., 1986; Rössler et al., 1999) but furthermore produced a female-like host plant searching behaviour. Schneiderman et al. found that gynandromorph females (females with a grafted male antenna) oriented towards the female sex pheromone (Schneiderman et al., 1986). This behaviour is exhibited by normal males but has not been observed in normal females because the antennae of *M. sexta* females lack ORCs responsive to the main sex pheromone components (Kaissling, 1989). In interspecific antennal imaginal disc transplantation experiments between *Heliothis virescens* and *Helicoverpa zea* males, both types of unilateral transplants continued to exhibit upwind antemotactic flight in a wind tunnel to the recipient pheromone blend (Vickers et al., 2003). In contrast with our results, the interspecific transplantation of only one antenna was not sufficient to produce a behavioural change.

We also observed that the flight tracks of normal females towards host plants were similar to those of male gynandromorphs that developed a feminised AL, i.e. a behavioural sequence involving initial random flight, anemotactic zig-zag flight that narrowed as the moth approached the odour source, diminished rate of forward advance or hovering and host plant contact. Thus, our results support the hypothesis (Schneiderman et al., 1986) that a common motor pathway is involved in mediating odour search behaviour in both males and females. Altogether, these results indicate that ORC identity dictates odour-specific modulated behaviour.

Finally, we tested whether ORCs confer odour selectivity to their postsynaptic targets in the ALs. Using intracellular recording and staining, we tested the odour responses of male gynandromorph AL neurons. We found that male gynandromorph projection neurons with arborisations in the induced LFGs respond strongly to the (+) enantiomer of linalool, as do latLFG projection neurons from normal females (Reisenman et al., 2004). The few projection neurons we could successfully label in the induced LFGs had dendritic arborisations in both LFGs. In one preparation, we found that a projection neuron with arborisations in the lateral-most part of the AL had an axonal process targeting the distinct protocerebral target area of MGC projection neurons. Thus, although the low numbers of projection neurons labelled do not allow us to make definitive conclusions about the role of ORCs in determining projection neuron morphology, it is possible that the morphological development of some projection neurons may occur independently of antennal afferent input, as previously suggested (Oland et al., 1990; Malun et al., 1994). This might be particularly true in the case of projection neurons in sexually dimorphic glomeruli, because those are the first glomeruli that form during development (Rössler et al., 1998). We found that projection neurons in sexually isomorphic glomeruli have morphologies and physiological responses similar to those of normal animals (see Reisenman et al., 2008).

In sum, our results show that the presence of female-specific olfactory structures in the AL is necessary and sufficient to mediate female-specific olfactory behaviour. Formation of female-specific glomeruli and projection neurons depends upon the ingrowth of female-specific ORCs. Furthermore, these ORCs instruct odour-specific behaviour and confer specific odour tuning to their glomerular targets.

LIST OF SYMBOLS AND ABBREVIATIONS

AL	antennal lobe
AN	antennal nerve
D	dorsal
L	lateral
latLFG	lateral female glomeruli
LC	lateral cluster
LFG	large female glomeruli
MC	medial cluster
MGC	macroglomerular complex
OL	optic lobe
OR	odds ratio
ORCs	olfactory receptor cells

REFERENCES

Ache, B. W. and Young, J. M. (2005). Olfaction: diverse species, conserved principles. *Neuron* **48**, 417-430.

Buck, L. B. (1996). Information coding in the vertebrate olfactory system. *Ann. Rev. Neurosci.* **19**, 517-544.

Christensen, T. A. and Hildebrand, J. G. (1987). Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta. J. Comp. Physiol. A* **160**, 553-569.

Fraser, A. M., Mechaber, W. L. and Hildebrand, J. G. (2003). Electroantennographic and behavioral responses of the sphinx moth *Manduca sexta* to host plant headspace volatiles. *J. Chem. Ecol.* **29**, 1813-1833.

Guerenstein, P., Christensen, T. A. and Hildebrand, J. G. (2004). Sensory processing of ambient-CO_2 information in the brain of the moth *Manduca sexta. J. Comp. Physiol. A* **190**, 707-725.

Hanson, F. E. and Dethier, V. G. (1973). Role of gustation and olfaction in food plant discrimination in the tobacco hornworm, *Manduca sexta. J. Insect Physiol.* **5**, 1019-1034.

Hansson, B. S., Christensen, T. A. and Hildebrand, J. G. (1991). Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta. J. Comp. Neurol.* **312**, 264-278.

Heinbockel, T., Christensen, T. A. and Hildebrand, J. G. (1999). Temporal tuning of odor responses in pheromone-responsive projection neurons in the brain of the sphinx moth *Manduca sexta. J. Comp. Neurol.* **409**, 1-12.

Hildebrand, J. G. and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Ann. Rev. Neurosci.* **20**, 595-631.

Hildebrand, J. G., Hall, L. M. and Osmond, B. C. (1979). Distribution of binding sites for ^{125}I-labeled α-bungarotoxin in normal and deafferented antennal lobes of *Manduca sexta. Proc. Natl. Acad. Sci. USA* **76**, 499-503.

Kaissling, K. E., Hildebrand, J. G. and Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth *Manduca sexta. Arch. Insect Biochem. Physiol.* **10**, 273-279.

King, J., Roche Christensen, T. A. and Hildebrand, J. G. (2000). Response characteristics of a identified, sexually dimorphic olfactory glomerulus. *J. Neurosci.* **20**, 2391-2399.

Loughrin, J., Hamilton-Kemp, T., Andersen, R. and Hildebrand, D. (1990). Headspace compounds from flowers of *Nicotiana tabacum* and related species. *J. Agric. Food. Chem.* **38**, 455-460.

Malun, D., Oland, L. A. and Tolbert, L. P. (1994). Uniglomerular projection neurons participate in early development of olfactory glomeruli in the moth *Manduca sexta. J. Comp. Neurol.* **350**, 1-22.

Mechaber, W. L., Capaldo, C. T. and Hildebrand, J. G. (2002). Behavioral responses of adult female tobacco hornworms, *Manduca sexta*, to host plant volatiles change with age and mating status. *J. Insect Sci.* **2**, 1-8.

Mori, K., Takahashi, Y. K., Igarashi, K. M. and Yamaguchi, M. (2006). Maps of odorant molecular features in the mammalian olfactory bulb. *Physiol. Rev.* **86**, 409-433.

Oland, L. A. and Tolbert, L. P. (1987). Glial patterns during early development of antennal lobes of *Manduca sexta*: a comparison between normal lobes and lobes deprived of antennal axons. *J. Comp. Neurol.* **255**, 196-207.

Oland, L. A. and Tolbert, L. P. (1988). Effects of hydroxyurea parallel the effects of radiation in developing olfactory glomeruli in insects. *J. Comp. Neurol.* **278**, 337-387.

Oland, L. A., Tolbert, L. P. and Mossman, K. L. (1988). Radiation – induced reduction of the glial population during development disrupts the formation of olfactory glomeruli in an insect. *J. Neurosci.* **8**, 353-367.

Oland, L. A., Orr, G. and Tolbert, L. P. (1990). Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. *J. Neurosci.* **10**, 2096-2112.

R Development Core Team (2005). A *Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org.

Raguso, R., Henzel, C., Buchmann, S. and Nabhan, G. (2003). Trumpet flowers of the Sonoran desert: floral biology of *Peniocereus* cacti and sacred *Datura*. *Internat. J. Plant Sci.* **164**, 877-892.

Reisenman, C. E., Christensen, T. A., Francke, W. and Hildebrand, J. G. (2004). Enantioselectivity of projection neurons innervating identified olfactory glomeruli. *J. Neurosci.* **24**, 2602-2611.

Reisenman, C. E., Christensen, T. A. and Hildebrand, J. G. (2005). Chemosensory selectivity of output neurons innervating an identified, sexually isomorphic olfactory glomerulus. *J. Neurosci.* **25**, 8017-8026.

Reisenman, C. E., Heinbockel, T. and Hildebrand, J. G. (2008). Inhibitory interactions among olfactory glomeruli do not necessarily reflect spatial proximity. *J. Neurophysiol.* **100**, 554-564.

Reisenman, C. E., Riffell, J. A. and Hildebrand, J. G. (2009). Neuroethology of oviposition behavior in the moth *Manduca sexta. Ann. New York Acad. Sci.* **1170**, 462-467.

Reisenman, C. E., Riffell, J. A., Bernays, E. and Hildebrand, J. G. (2010). Antagonistic effects of floral scent in an insect-plant interaction. *P. Roy. Soc. Lond. B.* In press.

Rospars, J. P. and Hildebrand, J. G. (1992). Anatomical identification of glomeruli in the antennal lobes of the male sphinx moth *Manduca sexta. Cell Tiss. Res.* **270**, 205-227.

Rospars, J. P. and Hildebrand, J. G. (2000). Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta. Chem. Senses* **25**, 119-129.

Rössler, W., Tolbert, L. P. and Hildebrand, J. G. (1998). Early formation of sexually dimorphic glomeruli in the developing olfactory lobe of the brain of the moth *Manduca sexta. J. Comp. Neurol.* **396**, 415-428.

Rössler, W., Randolph, P. W., Tolbert, L. P. and Hildebrand, J. G. (1999). Axons of olfactory receptor cells of transsexually grafted antennae induce development of sexually dimorphic glomeruli in *Manduca sexta. J. Neurobiol.* **38**, 521-541.

Sanes, J. S. and Hildebrand, J. G. (1976). Structure and development of antennae in the moth, *Manduca sexta. Dev. Biol.* **51**, 282-299.

Schneiderman, A. M., Matsumoto, S. G. and Hildebrand, J. G. (1982). Trans-sexually grafted antennae influence development of sexually dimorphic neurons in moth brain. *Nature* **298**, 844-846.

Schneiderman, A. M., Hildebrand, J. G., Brennan, M. M. and Tumlinson, J. H. (1986). Trans-sexually grafted antennae alter pheromone-directed behavior in a moth. *Nature* **322**, 801-803.

Schweitzer, E. S., Sanes, J. R. and Hildebrand, J. G. (1976). Ontogeny of elctroantennogram responses in the moth, *Manduca sexta. J. Insect Physiol.* **22**, 995-960.

Sparks, M. R. (1969). A surrogate leaf for oviposition by the tobacco hornworm. *J. Econ. Entomol.* **63**, 537-540.

Tolbert, L. P. and Hildebrand, J. G. (1981). Organization and synaptic ultrastructure of glomeruli in the antennal lobes of the moth *Manduca sexta*: A study using thin sections and freeze-fracture. *Proc. R. Soc. Lond. B, Biol. Sci.* **213**, 279-301.

Tolbert, L. P., Matsumoto, S. G. and Hildebrand, J. G. (1983). Development of synapses in the antennal lobes of the moth *Manduca sexta* during metamorphosis. *J. Neurosci.* **3**, 1158-1175.

Vickers, N. J., Poole, K. and Linn, C. E. (2003). Consequences of interspecies antennal imaginal disc transplantation on organization of olfactory glomeruli and pheromone blend discrimination. *J. Comp. Neurol.* **446**, 377-388.

Willis, M. A., Butler, M. A. and Tolbert, L. P. (1995). Normal glomerular organization of the antennal lobe is not necessary for odor-modulated flight in female moths. *J. Comp. Physiol. A* **176**, 205-216.

Yamamoto, R. T., Jenkins, R. Y. and McClusky, R. K. (1969). Factors determining the selection of plants for oviposition by the tobacco hornworm *Manduca sexta. Entomol. Exp. Appl.* **12**, 504-508.