

# Organization of the Olfactory System of Nymphalidae Butterflies

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## Abstract

Olfaction is in many species the most important sense, essential for food search, mate finding, and predator avoidance. Butterflies have been considered a microsmatic group of insects that mainly rely on vision due to their diurnal lifestyle. However, an emerging number of studies indicate that butterflies indeed use the sense of smell for locating food and oviposition sites. To unravel the neural substrates for olfaction, we performed an anatomical study of 2 related butterfly species that differ in food and host plant preference. We found many of the anatomical structures and pathways, as well as distribution of neuroactive substances, to resemble that of their nocturnal relatives among the Lepidoptera. The 2 species differed in the number of one type of olfactory sensilla, thus indicating a difference in sensitivity to certain compounds. Otherwise no differences could be observed. Our findings suggest that the olfactory system in Lepidoptera is well conserved despite the long evolutionary time since butterflies and moths diverged from a common ancestor.

**Key words:** *Aglais urticae*, antennal lobe, antenna, immunohistochemistry, morphology, *Polygonia c-album*

## Introduction

Butterflies are likely the most appreciated of all insect groups due to their beauty and bright colors, and the fact that even non-entomologists can easily identify many species. More than 10 000 species have been identified worldwide and they occupy most types of habitats. The earliest known fossil records of Lepidoptera dates at least 190 My (Grimaldi and Engel 2005) and the time for divergence of moths and butterflies dates at least 100 My (Braby et al. 2006; Wahlberg 2006). As opposed to most moths, butterflies have adopted an almost exclusively diurnal lifestyle.

Butterflies use a wide range of plants as hosts ranging from herbs to bushes and trees (Ackery 1988). A specific species may specialize on a limited array of host plants, whereas other species are more polyphagous. No matter what range they cover, a butterfly needs to find and evaluate a host plant that may be potentially good for oviposition. The insect also has to find food for itself and a partner to mate with. The senses involved in these tasks vary between insect groups. Olfaction is essential for survival and reproduction in most animals, and butterflies are likely not an exception. However, olfactory research in this suborder has been limited, as opposed to moths and other insects. One reason may be that butterflies have been believed not to rely on olfaction to the same extent as, for example, their

nocturnal relatives among the Lepidoptera but rather to depend on visual cues (Hambäck et al. 2007). Nevertheless, a few studies have shown behavioral responses to floral odorants (Andersson and Dobson 2003b; Omura and Honda 2009) and host plant-related odors (Ikeura et al. 2010). In addition, electroantennogram (EAG) recordings from different butterfly species have demonstrated responses to a large number of volatile compounds (Andersson and Dobson 2003a; Omura and Honda 2009), and recently odor-evoked activity was recorded in the primary central olfactory neuropil, the antennal lobe (AL), of 2 butterfly species by means of  $Ca^{2+}$  imaging (Carlsson et al. 2011).

A breakthrough in butterfly olfactory research was the recent sequencing of 2 butterfly genomes (Zhan et al. 2011; Dasmahapatra et al. 2012). A highly interesting annotation was the similarity of olfactory receptor genes, both numerically and qualitatively, to the nocturnal relatives, the moths. The similarity of the olfactory receptor repertoire is remarkable considering the different lifestyles of moths and butterflies and the evolutionary time since divergence from a common ancestor.

The major peripheral olfactory organ in insects is the antenna that contains numerous sensilla housing the olfactory sensory neurons (OSNs). The morphology of butterfly

antennae has been investigated in a few studies (Myers 1968; Grula and Taylor 1980; Odendaal et al. 1985). A characteristic feature of butterflies is the club-shaped antenna, that is, the distal segments of the flagellum, the bulb, is far thicker than the more basal segments. Another feature is the lack of sexual dimorphism, which likely reflects the fact that butterflies, as opposed to moths, are not known to communicate with long-distance female-emitted pheromones (Andersson et al. 2007).

Only few data are available concerning the anatomy and neuroarchitecture of the butterfly central olfactory system. In *Pieris rapae* (Rospars 1983), *Polygonia c-album*, *Aglaia urticae* (Carlsson et al. 2011), and the Monarch butterfly (Zhan et al. 2011; Heinze and Reppert 2012), the primary olfactory neuropil, the AL, consists of about 60 olfactory glomeruli, which is comparable with most studied moth species (Rospars 1983; Rospars and Hildebrand 1992; Berg et al. 2002; Masante-Roca et al. 2005; Kazawa et al. 2009; Varela et al. 2009). However, to our knowledge, only 2 neuroactive substances, gamma-aminobutyric acid (GABA) and serotonin, have been described in the olfactory system of butterflies (Dacks et al. 2006; Heinze and Reppert 2012).

Therefore, we performed a morphological study using scanning electron microscopy, neural tract tracing, and immunohistochemical experiments in 2 species of nymphalidae butterflies to start unravelling the architecture of the olfactory system. The 2 species, *A. urticae* and *P. c-album*, are common all over Europe and Asia and live sympatrically. However, their respective host plant range differs dramatically (Ackery 1988; Nylin 1988; Elbert 1993; Janz et al. 2001). Although *A. urticae* oviposits exclusively on stinging nettles, *P. c-album* has been found to lay eggs on a wide range of plants from several nonrelated families (Eliasson et al. 2005). Furthermore, *A. urticae* is basically a nectar feeder, whereas *P. c-album* preferentially feeds on rotten fruit and tree sap (Eliasson et al. 2005). The purpose of the study was to obtain an initial description of organization of the olfactory system of butterflies, and also to investigate whether differences in feeding and host plant specialization render observable morphological differences in the olfactory system. In addition to morphological features, we studied the distribution of GABA, nitric oxide, serotonin, and 3 neuropeptides in the olfactory neuropils. Finally, we discuss the butterfly olfactory system in an evolutionary perspective, specifically in relation to their closest relatives, the moths.

## Materials and methods

### Animals

Males and females of the Comma butterfly, *P. c-album* (Lepidoptera: Nymphalidae; Linnaeus), and the Small Tortoiseshell, *Aglaia urticae* (Lepidoptera: Nymphalidae; Linnaeus) were used in the study. The parental generations were caught in the vicinity of Stockholm, Sweden, and their

offspring was used. No permits were required in accordance with the Swedish principle of public access to the wilderness because the studied species are not protected or endangered. Prior to experiments, the butterflies were kept in cages at 25 °C, were allowed to mate, and had *ad libitum* access to sugar solution.

### Scanning electron microscopy

The antennae, still attached to the head, were fixed in 2.5% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS) for at least 1 h at room temperature. After repeated rinsing in PBS, the samples were dehydrated in an increasing series of ethanol (1 50%, 2 70%, 1 90%, 1 95%, and 2 absolute) until they were ready for the final critical point drying (CPD). After CPD, the antennae were severed from the head, fixed on a stub, and sputtered with gold before scanning with a Zeiss Supra 35-VP (Carl Zeiss SMT) scanning electron microscope, equipped with a field emission gun.

### Tracing experiments

A butterfly was placed in a 1000 µl pipette tip with the tip cut open to fit the head. The protruding head at the narrow end was fixed in this position with dental wax. A window was cut in the head capsule between the compound eyes, and the tissue covering the brain was removed to uncover the ALs, whereupon physiological saline (Christensen and Hildebrand 1987) was immediately applied to the exposed brain. Crystals of rhodamine dextran with biotin (3000 MW, Microruby, D7162; Invitrogen) were inserted into the AL using a steel micro pin. During insertion, the saline was temporarily removed to prevent dissolving the dye. For tracing antennal neurons to the AL, we applied crystals of fluorescein dextran with biotin (3000 MW, Microemerald, D7156; Invitrogen) to the fresh wound of a cut antenna. The dye was allowed to diffuse overnight at 4 °C. Subsequently, the brain was dissected and fixed over night in 4 °C in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB). The brains were put in 20% sucrose solution in PBS overnight and subsequently frozen at -23 °C and cut at 50 µm on a cryostat (Leica CM 1850).

### Immunocytochemistry

Brains of butterflies were dissected in 0.1 M PB and fixed either for 4 h at room temperature or overnight at 4 °C in 4% PFA in PB. After careful rinsing in PB, the brains were pre-incubated overnight in 5% normal goat serum in PBS with 0.25% Triton-X (PBS-Tx). For whole mount, brain tissue was incubated for 48–96 h in primary antisera. For sections, we used the same method as above and tissue was incubated for 48 h in primary antisera.

For detection of antisera, we used Alexa 488/546-tagged secondary antibodies (Invitrogen). Secondary antibodies were used at a 1:1000 dilution. Whole mount preparations

were dehydrated in a series of increasing ethanol concentration (30–100%), and finally cleared and mounted in methyl salicylate. Sections were embedded in 80% glycerol in PBS.

### Antibodies

#### *Gamma-aminobutyric acid*

For detection of GABA, we used a commercially available rabbit antiserum to GABA (Sigma; A2052) that was raised to GABA–bovine serum albumin conjugate and then affinity purified (Hamasaka et al. 2005). The antibody was used at 1:1000 dilution.

#### *Tachykinin-related peptides*

To detect tachykinin-related peptides (TRPs), we used a rabbit antiserum raised against the *Leucophaea maderae* TRP, LemTRP-1, produced by immunization with the full peptide sequence (APSGFLGVRamide) coupled to bovine serum albumin with carbodiimide, code K 9836 (Winther and Nässel 2001). The antibody was used at 1:4000 dilution.

#### *Myoinhibitory peptide*

Antiserum to myoinhibitory peptide (MIP) was generated in rabbits to MIP of *Periplaneta americana* (sequence GWQDLQGGWamide) coupled to thyroglobulin (Predel et al. 2001), provided by C. Wegener, Würzburg, Germany. The antibody was used at 1:4000 dilution.

#### *Allatostatin A*

Antiserum to allatostatin A (AstA) was raised in rabbit against synthetic *Diploptera punctata* allatostatin I coupled to bovine thyroglobulin with glutaraldehyde (Vitzthum et al. 1996), provided by HJ Agricola, Jena, Germany. The antibody was used at 1:4000 dilution.

#### *Nitric oxide synthase*

The universal nitric oxide synthase (uNOS) antiserum (1:200, Affinity Bioreagents) was raised in rabbit against a conserved sequence of NOSs (KRYHEDIFG). The antiserum labels proteins of similar expected molecular weights in a moth (Gibson and Nighorn 2000).

#### *Serotonin (5HT)*

To detect serotonin, we used a rabbit polyclonal antibody (Sigma; S-5545) at a dilution of 1:1000 (Hamasaka and Nässel 2006).

#### *Synapsin*

For counterstaining of synaptic neuropil, we used mouse monoclonal anti-synapsin (Klagges et al. 1996), at a dilution of 1:20 (Developmental Studies Hybridoma Bank). To stain

neurons in sections of the antennae, we used 3% Alexa Fluor 546 phalloidin (Invitrogen) and for nuclear counterstaining we used TOTO-3 (Invitrogen) at a dilution of 1:1000.

### NADPH diaphorase staining

NADPH diaphorase staining can be used as a marker for nitric oxide signalling cells because it frequently colocalizes with NOS (Stern et al. 2010). Sections of brains or antennae were washed twice in PBS-Tx and transferred to staining solution (2 mg nitroblue tetrazolium and 2 mg NADPH in 5 ml PBS-Tx) for 60–90 min at room temperature in the dark. Staining was stopped by washes in distilled water. Sections were subsequently embedded in 80% glycerol and viewed under a wide-field microscope.

### Image processing

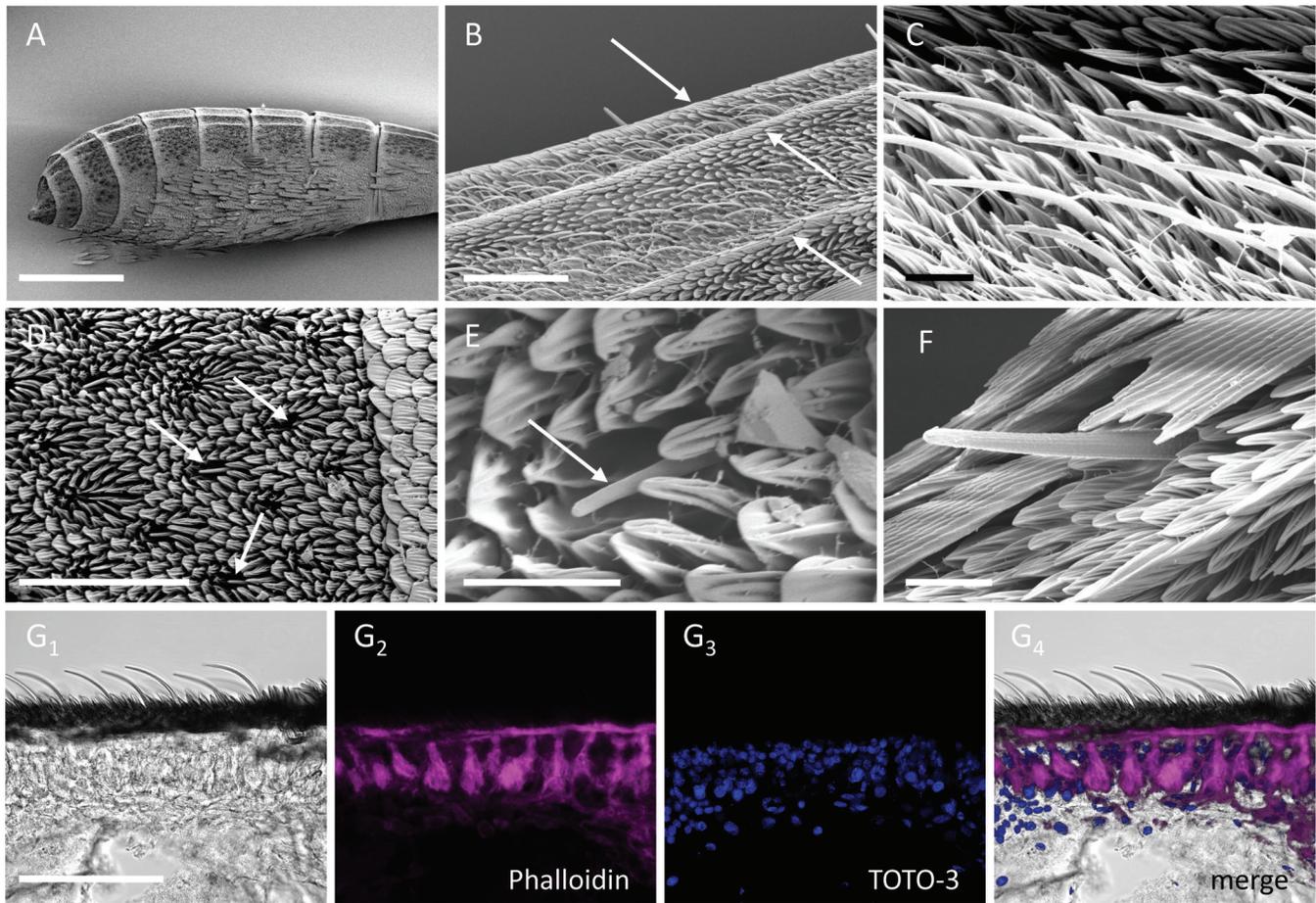
Whole mount preparations or sections were imaged with a Zeiss LSM 510 META (Zeiss) confocal laser scanning microscope. Images were subsequently processed with Zeiss LSM Software and edited for contrast and brightness in Adobe Photoshop CS4 Extended (vers. 11.0). For presentation, we used either projection images of stacked optical sections or single images.

Three-dimensional reconstructions of confocal stacks were generated using texture-based volume rendering with AMIRA 4.1 software (Mercury Computer Systems GmbH).

## Results

### Antennal morphology

The antennae of both species and sexes consisted of a flagellum of 34 segments (flagellomeres), including the bulb (Figure 1A). The bulb comprises the distal 10–12 segments. Using the classification terminology of sensilla from an earlier study (Odendaal et al. 1985) in another nymphalidae species, *Euphydryas editha*, we could identify 4 types of sensilla, types I, II, III, and IV. A common feature was the presence of 3 longitudinal ridges separating 2 grooves running from the base to the tip of the antenna (Figure 1B). In the 2 grooves, we found all the type II sensilla (Figure 1C) that were curved and about 30 µm long. The number of sensilla type II was significantly higher in *P. c-album* than in *A. urticae* ( $P < 0.01$ , ANOVA followed by Tukey's multiple comparison test,  $n = 3$ ). However, there was no difference between the sexes within a species. A third type (III) was found basically on either side of the grooves along the antenna (Figure 1D and E). This sensillum was shorter than the former (about 10 µm), straight and located in pits sheltered by a ring of scales. Type I sensillum is straight and about 40 µm long (Figure 1F). These sensilla were mainly found at the basal end of each segment. A fourth type of sensillum (IV) had a whip-like appearance and occurred with low frequency mainly on the more basal segments of the



**Figure 1** Scanning electron microscope images of antennal structures. (A) The distal bulb of an antenna from a female *A. urticae*. Scale bar = 500  $\mu\text{m}$ . (B) The 2 grooves separated by 3 ridges (arrows) with type II sensilla in a female *P. c-album*. Scale bar = 50  $\mu\text{m}$ . (C) A close-up of sensilla type II in a female *A. urticae*. Scale bar = 10  $\mu\text{m}$ . (D) Sensilla type III hidden in scale-framed pits (arrows) on the bulb of a female *A. urticae*. Scale bar = 50  $\mu\text{m}$ . (E) A close-up of a sensillum type III in a female *A. urticae*. Scale bar = 5  $\mu\text{m}$ . (F) A sensillum type I in a female *P. c-album*. Scale bar = 10  $\mu\text{m}$ . (G) Cryosections of antennae of a female *P. c-album*. Transmission image (G1) shows a row of sensilla type II, staining with phalloidin (magenta) and the nuclear marker TOTO-3 (blue). Scale bar = 100  $\mu\text{m}$ .

flagellum. The latter sensillum was in general hidden beneath the scales, which made an accurate quantification unfeasible. In Table 1, the number of the different sensilla (types I–III) in both species and sexes are summarized. Whereas type I was evenly distributed over the antenna, types II and III had the highest density on the medial segments with numbers decreasing toward the basal and distal ends.

Cryosections of the antennae were stained with Alexa Fluor 546 phalloidin and a nuclear dye (TOTO-3). In Figure 1G, large neuronal cells (magenta) are tightly connected to type II sensilla. In sections with no sensilla, we did not observe any staining of cell bodies with phalloidin. Generally, there were about twice as many stained neurons as sensilla, indicating that each sensillum contains >1 neuron.

### Tracing experiments

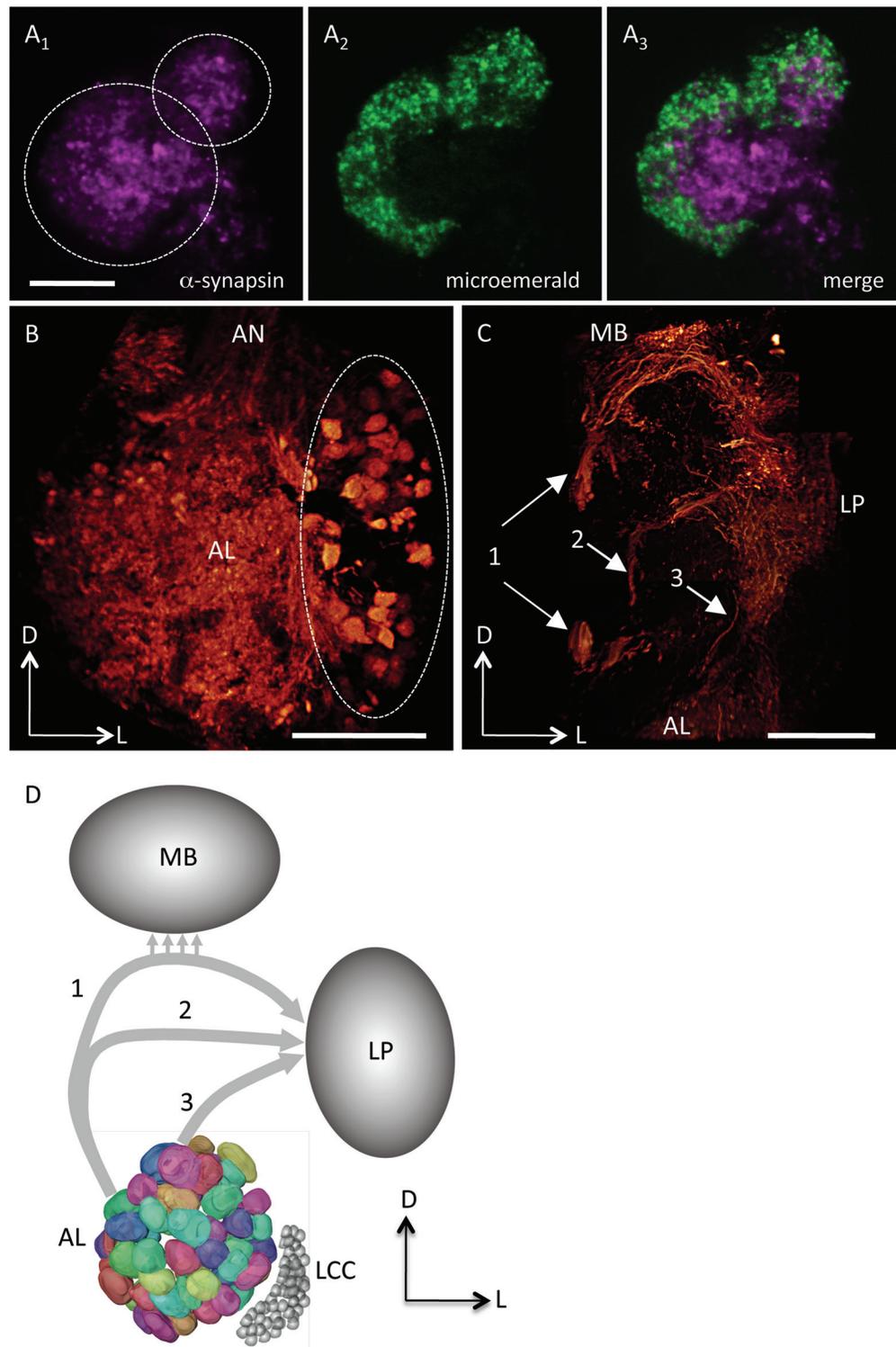
A neural tracer (Microemerald) inserted into a fresh wound of a cut antenna revealed dense innervation in the

outermost layer of individual glomeruli in the ipsilateral AL of both species (Figure 2A). By mechanical damage of AL neurons and insertion of a neural tracer (Microruby),

**Table 1** Estimation of numbers of flagellum segments and sensilla in male and female *P. c-album* and *A. urticae*

	<i>P. c-album</i> (female)	<i>P. c-album</i> (male)	<i>A. urticae</i> (female)	<i>A. urticae</i> (male)
Segments	34 $\pm$ 0	34 $\pm$ 0	34 $\pm$ 0	34 $\pm$ 0
Sensillum type I	115 $\pm$ 3	117 $\pm$ 1	102 $\pm$ 4	111 $\pm$ 6
Sensillum type II	2083 $\pm$ 117	2113 $\pm$ 124	1455 $\pm$ 105	1415 $\pm$ 7
Sensillum type III	2746 $\pm$ 155	2899 $\pm$ 202	2432 $\pm$ 140	2639 $\pm$ 98

Mean  $\pm$  SEM,  $n = 3$ . The number of type II sensilla differ significantly between species but not between sexes of same species ( $P < 0.01$ , ANOVA followed by Tukey's multiple comparison test,  $n = 3$ ).



**Figure 2** Tracing experiments. **(A)** Two glomeruli in the AL of a female *P. c-album* visualized by synapsin labelling (magenta). Innervation of OSNs (green) is seen in the outermost layer of the glomeruli. Scale bar = 20  $\mu$ m **(B)** Insertion of a neural tracer in the AL of a female *A. urticae* reveals a large lateral cluster of cell bodies (dashed oval). Voltex reconstruction from a confocal image stack. AN, antennal nerve; D, dorsal, L, lateral. Scale bar = 100  $\mu$ m. **(C)** ACTs and termination sites in a female *A. urticae*. Overlay of voltex reconstructions from 3 consecutive confocal stacks reveals 3 different tracts (1–3) leaving the AL. Tracts 2 and 3 project directly to the LP, whereas tract 1 first contacts the calyces of the mushroom bodies before finally targeting the LP. Scale bar = 100  $\mu$ m. **(D)** Schematic drawing of the 3 tracts and the termination sites. D, dorsal, L, lateral. The AL is a 3D surface reconstruction of a confocal stack of anti-synapsin stained glomeruli in a female *A. urticae* (AMIRA 4.1). The colors of glomeruli are arbitrary. LCC, lateral cell cluster.

we anterogradely traced axons of projection neurons (PN) to terminals in the protocerebrum. First of all, the staining revealed a large cluster of cell bodies lateral to the AL (Figure 2B). In addition, 3 different antennocerebral tracts (ACTs) leaving the AL could be identified as shown in an overlay of 3 optical stacks (Figure 2C). The most lateral ACT projects directly to an area in the lateral protocerebrum. The second ACT split up from a thicker tract bundle and projects toward the dorsal protocerebrum before turning lateral and terminating in the lateral protocerebrum. The third ACT was the second part of the thicker tract bundle and this tract continued along the brain midline and terminated first in the calyx of the mushroom body and then continued to the lateral protocerebrum. Figure 2D shows a schematic drawing of the 3 tracts and the axon termination sites.

#### NOS and NADPH as markers for OSNs in the antenna

In cryosections of antennae from both species, we observed stained cell bodies with both anti-uNOS and NADPHd (Figure 3A–C). These cell bodies were found along the entire

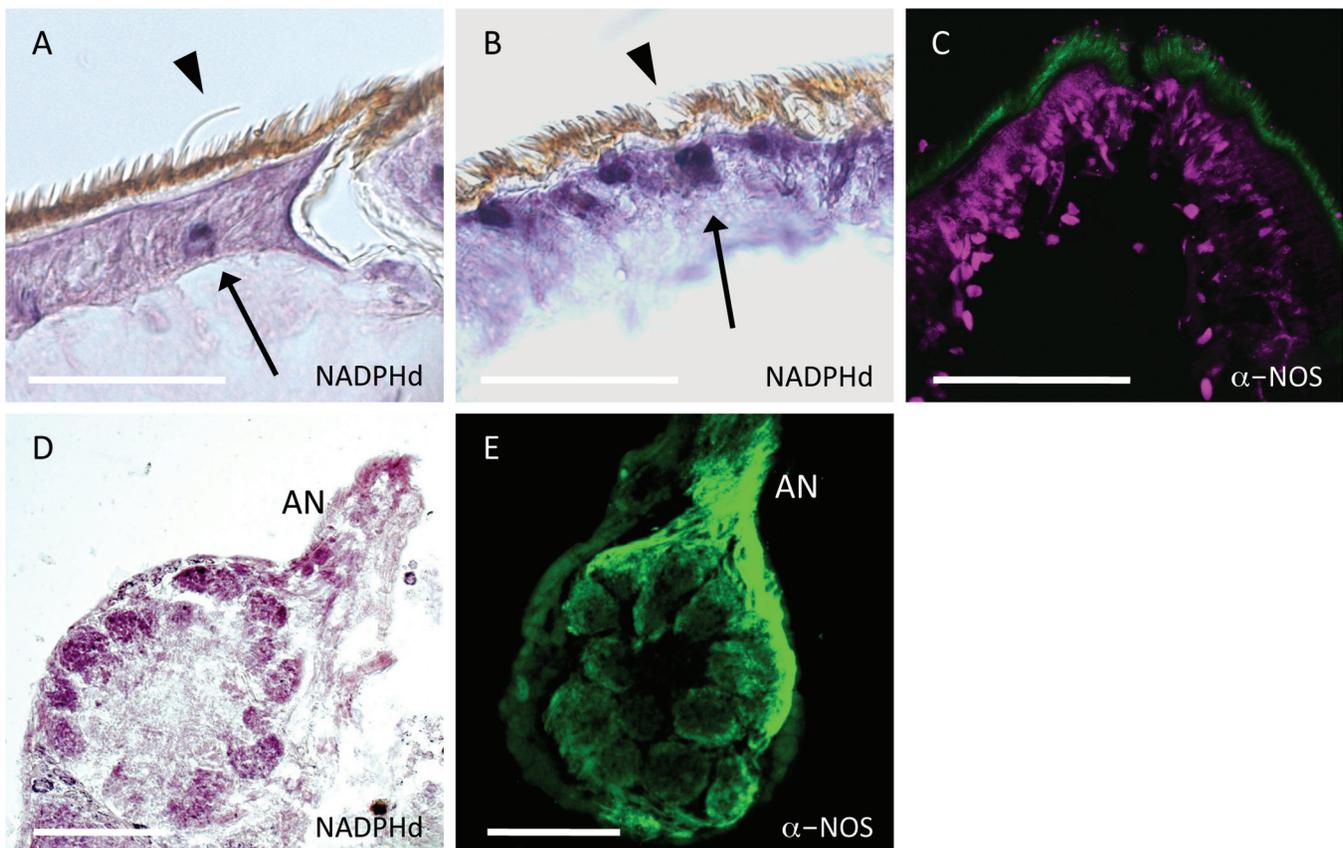
flagellum, including the bulb. Furthermore, the cell bodies were mainly found in close conjunction with sensilla types II and III (arrows, Figure 3A and B). Usually, 2 cell bodies were clustered together, indicating innervation of 2 sensory neurons per sensillum.

#### NOS and NADPH in the AL

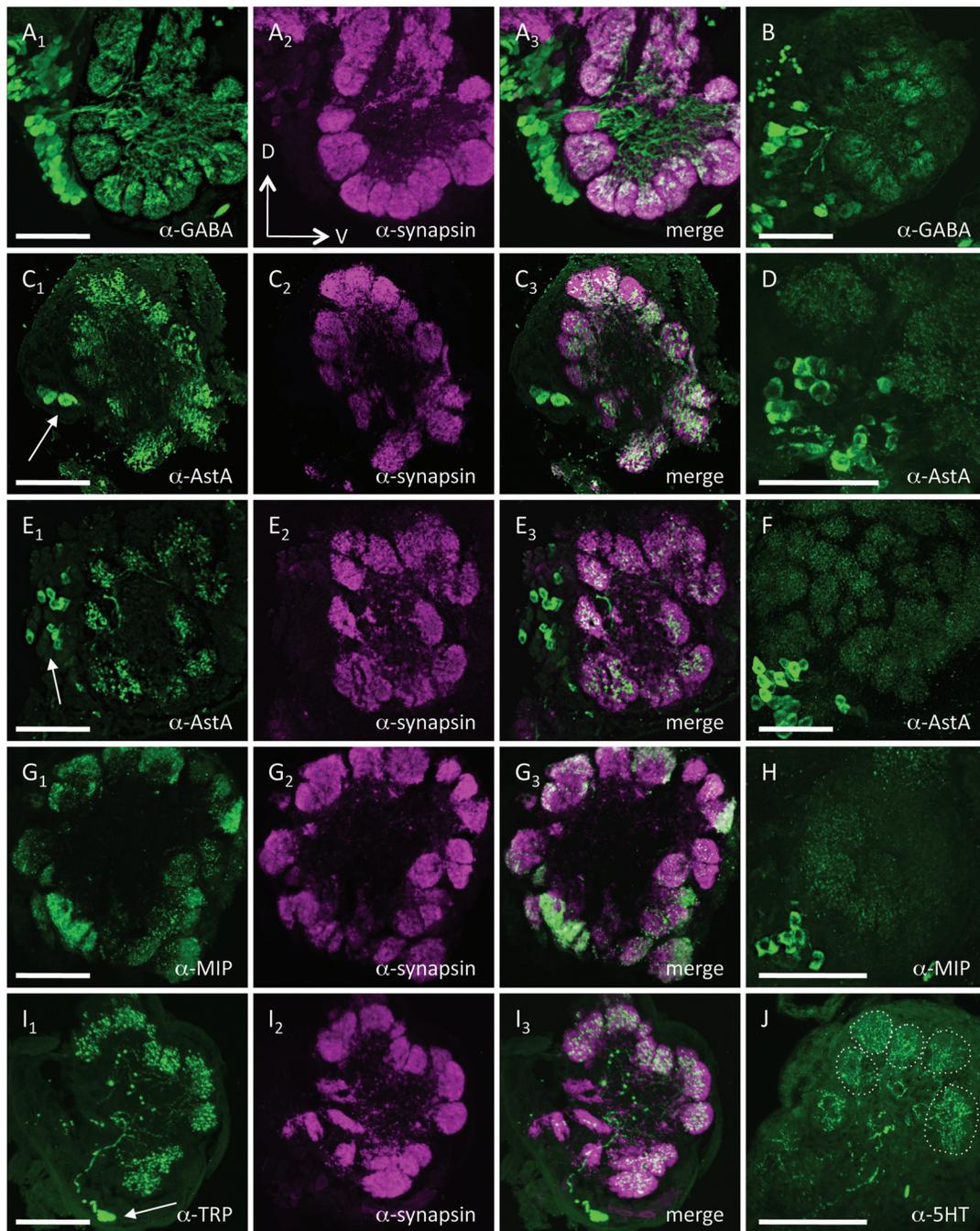
NADPHd staining was distributed in all glomeruli in the ALs of both species (Figure 3D). No labelled cell bodies were observed in the vicinity of the AL, indicating that axons stem mainly from OSNs. The antibody to NOS also detected expression within neurites in the AL, which mainly innervated the outermost layer of glomeruli (Figure 3E). Neither with anti-NOS did we detect any stained somata in the AL cell clusters.

#### Gamma-aminobutyric acid

GABA immunoreactive neurons were observed densely innervating the agglomerular central neuropil of the AL and the core of all glomeruli (Figure 4A and B and Table 2). The



**Figure 3** Nitric oxide labelling in OSNs and the AL. (A) NADPHd labelling of a cell body (arrow) in conjunction with a type II sensillum (arrow head) of a male *P. c-album*. (B) NADPHd labelling of a cell body (arrow) in conjunction with a type III sensillum (arrow head) of a male *P. c-album*. (C) Anti-NOS immunolabelling of cell bodies in the club of the antenna (magenta). The cuticle is shown as green (autofluorescence) of a female *P. c-album*. (D) NADPHd labelling in the AL of a male *P. c-album*. Staining is seen in most or all glomeruli and the antennal nerve (AN). (E) Anti-NOS immunolabelling in the AL of a female *P. c-album*. Staining is particularly strong in the antennal nerve (AN) and in the outermost layers of glomeruli. Scale bars = 100 µm.



**Figure 4** Distribution of neuroactive substances in AL neurons. **(A)** Anti-GABA immunolabelling of a female *P. c-album* reveals dense innervation in the central aglomerular neuropil as well as the core of all glomeruli. Immunolabelling with anti-GABA (green), anti-synapsin (magenta), and merged. Maximum projection of optical sections from a cryostat-sectioned AL. **(B)** Maximum projection of a confocal image stack of anti-GABA staining in the AL from a whole mount preparation of a female *P. c-album*. **(C)** Immunolabelling with anti-AstA (green), anti-synapsin (magenta), and merged of a female *P. c-album*. Single optical section from a cryostat-sectioned AL. The arrow points at AstA cell bodies in the lateral cell cluster. **(D)** Maximum projection of a confocal image stack of anti-AstA staining in the AL from a whole mount preparation of a female *P. c-album* showing the cluster of cell bodies in the lateral cell cluster. **(E)** Immunolabelling with anti-AstA (green), anti-synapsin (magenta), and merged of a female *A. urticae*. Maximum projection of optical sections from a cryostat-sectioned AL. The arrow points at AstA cell bodies in the lateral cell cluster. **(F)** Maximum projection of a confocal image stack of anti-AstA staining in the AL from a whole mount preparation of a female *A. urticae* showing the cell bodies in the lateral cell cluster. **(G)** Immunolabelling with anti-MIP (green), anti-synapsin (magenta), and merged of a female *P. c-album*. Single optical section from a cryostat-sectioned AL. **(H)** Maximum projection of a confocal image stack of anti-MIP staining in the AL from a whole mount preparation of a female *P. c-album* showing the cell bodies in the lateral cell cluster. **(I)** Immunolabelling with anti-TRP (green), anti-synapsin (magenta), and merged of a female *P. c-album*. Single optical section from a cryostat-sectioned AL. The arrow points at a TRP cell body in the lateral cell cluster. **(J)** Immunolabelling with anti-serotonin in the AL of a female *P. c-album*. Single optical section from a cryostat-sectioned AL. Glomeruli are outlined. D, dorsal, V, ventral. Scale bars = 100  $\mu\text{m}$ .

somata were found in a large lateral cell cluster (soma diameter 15–20  $\mu\text{m}$ ). The total number of GABA immunoreactive cells amounted >100 (both species and sexes). We did not detect any axons leaving the AL, indicating that most (or all) GABAergic neurons are local interneurons (LNs). The number of cells is estimated from both cryosections and whole mounts ( $n \geq 5$  specimens of each sex and species, which also applies for all estimations below).

### Allatostatin A

Allatostatin A (AstA) immunoreactive neurons were found in the AL with sparse innervation in the central glomerular neuropil and arborizations in the core of all (or most) glomeruli (Figure 4C–F). About 35 cell bodies were clustered in a ventro-lateral group (see Table 2, soma diameter 15–20  $\mu\text{m}$ ). No axons were observed leaving the AL, thus indicating that the neurons were LNs. For comparison, we show images of distribution in both species.

### Myoinhibitory peptide

About 15 laterally located MIP immunoreactive cells (soma diameter 15–20  $\mu\text{m}$ ) were observed (both species and sexes, Figure 4G and H and Table 2). These cells particularly innervated the outermost layer of all (or most) glomeruli in the AL and only sparsely the central glomerular neuropil. The lack of axons leaving the AL indicates that the neurons were LNs.

### Tachykinin-related peptides

There were less immunoreactive cells for TRP in the AL than for the other tested neuropeptides (Figure 4I and Table 2). Only about 5 cells (soma diameter 15–20  $\mu\text{m}$ ) in the lateral cell cluster could be observed (both species and sexes). Innervation in the AL was global (most or all glomeruli) but sparse in the glomerular central neuropil and core of glomeruli. Also these neurons were likely LNs based by the fact that no axons leaving the AL were observed.

For GABA, AstA, TRP, or MIP immunostainings, we did not find any significant differences in the number of cell bodies between species and sexes (see Table 2).

### Serotonin (5HT)

Serotonin immunoreactive neurons were found to sparsely innervate the ALs of both species (Figure 4J). The distribution was uneven in that more dorsally located glomeruli received more arborizations. We could not localize any cell bodies in the lateral cell cluster and neither did we observe any staining in the antennal nerve indicating extrinsic origin. Staining was, however, observed in several other areas of the brain, but we could not identify the origin of the cells innervating the AL. No difference between sexes and species were observed.

**Table 2** Summary of immunohistochemical experiments

Transmitter	<i>P. c-album</i> (female)	<i>P. c-album</i> (male)	<i>A. urticae</i> (female)	<i>A. urticae</i> (male)
Nitric oxide	OSN	OSN	OSN	OSN
GABA	LN, >100	LN, >100	LN, >100	LN, >100
AstA	LN, 34 $\pm$ 2	LN, 35 $\pm$ 1	LN, 36 $\pm$ 2	LN, 35 $\pm$ 2
TRP	LN, 5 $\pm$ 0	LN, 5 $\pm$ 0	LN, 5 $\pm$ 0	LN, 4 $\pm$ 1
MIP	LN, 14 $\pm$ 2	LN, 15 $\pm$ 1	LN, 14 $\pm$ 2	LN, 12 $\pm$ 1
Serotonin	EN	EN	EN	EN

The localization in the antennal lobe and estimated cell body numbers of each transmitter for both species and sexes. Estimations were made from both sections and whole mount preparations of at least 5 specimens of each species and sex. OSN, olfactory sensory neuron; LN, local interneuron; EN, extrinsic neuron. Mean  $\pm$  SEM. No significant differences between sexes and species.

## Discussion

Butterflies have been an utterly neglected insect group in olfactory research, probably due to the presumption that these animals are microsmatic and mainly rely on visual guidance. In our research group, we are primarily interested in the evolution of host plant specialization in butterflies. This study is part of a project where we try to entangle the mechanisms behind host plant choice and whether differences and limitations in sensory information processing can help explain the evolution of host plant specialization.

In this study, we describe the olfactory system of 2 phylogenetically related butterfly species with diverging host plant range and feeding preferences. Both species possess an olfactory system that in many respects resembles that of moths. However, the system is numerically reduced compared with most moth species studied, with substantially fewer olfactory sensilla (and thus OSNs). Moreover, the butterflies also possessed fewer peptidergic LNs than moths (Utz and Schachtner 2005; Berg et al. 2007; Utz et al. 2007).

The 2 species differ in their ability to behaviorally respond to plants, that is, *A. urticae* is only attracted to stinging nettles, whereas *P. c-album* seemed not to be able to make fine-tuned decisions between different combinations of host plants (Schäpers et al. submitted). In a previous optophysiological study of odor-evoked activity in the AL of *A. urticae* and *P. c-album*, we showed that the 2 species generally responded similarly to the tested stimuli (Carlsson et al. 2011). However, we found species-specific differences in responses to stinging nettle, the sole host plant for *A. urticae*. In the latter species, the response to stinging nettle differed more from responses to other green plants than it did in *P. c-album*. Furthermore, we found both qualitative and quantitative differences in responses to 2 green leaf volatiles (ubiquitously emitted from vegetative parts of plants) that may at least partly explain differences in attraction between the species. The purpose of this study was, therefore, to investigate potential morphological differences across the species

along with obtaining knowledge of the butterfly olfactory system in general.

The only difference between the 2 species we could observe was that both male and female *P. c-album* have more sensilla than *A. urticae*. Especially, the number of long curved sensilla (type II) differed between the species (almost 50% more in *P. c-album* than *A. urticae*). These sensilla were exclusively found in 2 longitudinal grooves separated by 3 ridges running along the entire antenna. This morphological characteristic has been previously described in another nymphalidae butterfly, *Danaus gilippus* (Myers 1968). In addition to the investigated species, we also examined the antennal structure of 3 other nymphalid species (*Vanessa atalanta*, *Inachis io*, and *Nymphalis antiopa*, data not shown), all of which had the groove-like structures with type II sensilla. This structure is, however, not present in the nymphalid species, *Euphydryas editha* (Odendaal et al. 1985) and is thus not a ubiquitous butterfly feature. In the latter butterfly, sensilla type II are instead located in shallow dishes similar to what has been observed in Pieridae butterflies (Grula and Taylor 1980). The lack of sexual dimorphism in the species investigated in this study likely reflects the fact that butterflies are not known to communicate with long distance pheromones (Andersson et al. 2007).

So, do these sensilla have an olfactory function? Most electrophysiological studies of antennal responses to odors in butterflies have been performed by EAG and not at the level of individual sensilla (Andersson and Dobson 2003a; Omura and Honda 2009). Den Otter et al. (1980) made recordings from individual cells from the antennae of *Pieris brassicae*. The “surface-contact” technique used did not allow identification of sensillum type. However, because recordings were done from the shallow dishes where type II sensilla are found, we may assume that at least these sensilla are housing OSNs. Type II sensilla are very likely identical to sensilla basiconica in moths, which have been demonstrated in a number of studies to be olfactory (Jönsson and Anderson 1999; Kalinová et al. 2001). Moreover, type III sensilla anatomically resemble sensilla coeloconica (Keil 1999), which in moths have been shown to respond to odors (Pophof 1997). Somata immunoreactive to a NOS antibody and detected by NADPHd staining were observed in large number in the antenna, and the somata were often seen in close association with the sensilla types II and III. This further indicates an olfactory function of neurons in these sensilla because anti-NOS and NADPH staining was mainly seen in the antennal nerve and the outermost layer of glomeruli in the AL. Furthermore, nitric oxide has been shown to act as an unconventional transmitter in OSNs together with acetylcholine in moths (Collmann et al. 2004). In some nonlepidopteran species, however, the NOS expressing neurons are partly or exclusively interneurons (Homberg and Müller 1999). Thus, the majority of antennal sensilla, that is, types II and III, likely have an olfactory function. In contrast, the large sensillum type I seems identical to sensilla

chaetica in other insects (Keil 1999). This sensillum type has been shown to house gustatory receptor neurons in the moth *Heliothis virescens* (Jørgensen et al. 2007).

In *Drosophila*, it has been demonstrated that sensilla basiconica (resemble the type II sensilla in the butterflies) house OSNs expressing 7-transmembrane ORs (Vosshall and Stocker 2007). In contrast, sensilla coeloconica house neurons expressing ionotropic glutamate-like receptors (IRs) (Benton et al. 2009). The IRs are believed to be the most evolutionarily ancient of chemosensory receptors present across animal, plant, and prokaryotic genomes (Silbering and Benton 2010). IRs may be responsible for detection of evolutionarily ancient volatiles, whereas factors such as the angiosperm radiation (and a whole new class of volatile compounds) and evolution of pheromone communication may have been driving forces for a need to expand the OR repertoire. It is interesting to note that the number of sensilla coeloconica (type III, possibly housing IRs) seems to be well conserved within Lepidoptera, whereas other types of sensilla (possibly housing ORs) vary considerably between species and sexes (Keil 1999).

The ALs of the 2 studied species consist of 60–65 glomeruli (Carlsson et al. 2011), which is similar to the Monarch butterfly (Zhan et al. 2011) and *Pieris brassicae* (Rosparis 1983). Interestingly, this number is also similar to moths but not to other insect orders (Anton and Homberg 1999), thus further consolidating the evolutionary conservation of the olfactory system within Lepidoptera. The glomerular number is likely mirroring the number of olfactory receptor types (Zhan et al. 2011), that is, each functional type of OSN (housing a specific receptor) converges on an individual glomerulus. The higher number of sensilla in *P. c-album* may thus rather reflect an increased sensitivity, due to more OSNs of each type, than more types of OSNs. In a previous study, we showed that *P. c-album* indeed had a lower threshold for the green leaf volatile 1-hexanol than *A. urticae* (Carlsson et al. 2011).

The AL consists of OSN axon terminations synapsing with higher order neurons, PNs and LNs. Tracing of axons from the antenna revealed dense arborization of the OSNs in the outermost layer of the innervated glomeruli in the AL, as has been observed in moth species but not in, for example, cockroaches (Anton and Homberg 1999). Insertion of a neural tracer in the AL revealed a large lateral cluster of cell bodies, likely belonging to both LNs and PNs. In an attempt to unravel the neuroactive substances these neurons use for signalling, we performed immunohistochemical experiments. First of all, we found GABA to be expressed in a large number (at least 100) AL neurons, most of which seem to be LNs. The branches of the GABAergic neurons innervated the entire lobe and densely the core of all glomeruli. This inhibitory transmitter is ubiquitous in insect LNs and was recently described in another butterfly, *Danaus plexippus* (Heinze and Reppert 2012). GABA acts on 2 different receptor types, a G-protein-coupled metabotropic receptor

(GABA-B) for slow modulatory action and an ionotropic receptor (GABA-A) for fast transmission (Olsen and Wilson 2008). The function of these in the olfactory system has been demonstrated in a number of studies in different insect species. Although GABA emitted from LNs through the GABA-A is shaping odor-evoked spatiotemporal postsynaptic patterns (MacLeod and Laurent 1996; Stopfer et al. 1997; Wilson and Laurent 2005), the GABA-B is also housed in OSNs and GABA thus mediates a presynaptic gain control in these neurons (Root et al. 2008).

In addition to GABA, we tested antibodies against 3 different neuropeptides that have been shown to be expressed in other insect ALs (Homberg and Müller 1999; Utz and Schachtner 2005; Berg et al. 2007; Carlsson et al. 2010). AstA was first discovered as a peptide inhibiting juvenile hormone production in the corpora allata of the cockroach *D. punctata* (Woodhead et al. 1989). AstA was the most widely expressed of the peptides we investigated. About 35 LNs expressed AstA. In moths, about 50 AstA expressing LNs have been observed (Utz and Schachtner 2005; Berg et al. 2007). On the other hand, in *Drosophila* only 3 LNs express AstA (Carlsson et al. 2010), whereas in cockroaches and locusts AstA is expressed in centrifugal feedback neurons (Homberg and Müller 1999). Currently, there is no known function of AstA in the olfactory system. In the gustatory system, on the other hand, it was recently demonstrated that AstA acts as a satiety factor that reduces the motivation to feed (Hergarden et al. 2012).

A second type of neuropeptide, MIP, was found to be expressed in about 15 LNs in the AL. In comparison, *Manduca sexta* has about 60 AL neurons expressing MIP (Utz et al. 2007), and *Drosophila* has about 10–15 LNs (Carlsson et al. 2010). This neuropeptide is frequently referred to as allatostatin B because it was originally identified in crickets where it has an allatostatic function (Lorenz et al. 1995). In other insects, it is usually myoinhibitory (Blackburn et al. 1995, 2001; Schoofs et al. 2001) or involved in regulation of ecdysis behavior (Williamson et al. 2001; Kim et al. 2006; Santos et al. 2007). There is currently no known function of MIP in the olfactory system of any species.

A third type of neuropeptide, TRP, is expressed in only about 5 LNs in both species. Also in the moths *Heliothis virescens* and *Manduca sexta*, the number of TRP immunoreactive neurons is comparatively small (Berg et al. 2007). In other insect orders, however, a far larger number of cells have been observed. Even the otherwise reduced olfactory system of *Drosophila* houses about 20 TRP immunoreactive LNs (Ignell et al. 2009; Carlsson et al. 2010). Recently, it was demonstrated that tachykinin plays a functional role in olfactory processing in *Drosophila* (Winther et al. 2006; Ignell et al. 2009). The peptide acts in presynaptic gain control of OSN output. Furthermore, knockdown or overexpression of the tachykinin receptor in OSNs resulted in altered olfactory behavior (Ignell et al. 2009).

Prior to this study, the only transmitter that had been described in the butterfly olfactory system, apart from GABA, is serotonin (Dacks et al. 2006; Heinze and Reppert 2012). This monoamine is expressed in a single bilateral neuron connecting the ipsilateral AL with the contralateral AL in all insect species investigated (Dacks et al. 2006). We can confirm that serotonin is also expressed in the ALs of the 2 species studied here, but we were not able to localize the cell bodies of these neurons. However, judging by the very sparse innervation in the ALs, it seems likely that it is the same type of bilateral neurons found in other species. Serotonin has been shown to enhance sensitivity in PNs in an odor-specific manner (Dacks et al. 2009). Furthermore, serotonin is involved in postmating dietary switch in *Drosophila*, and thus it likely regulates the sensitivity to related volatiles (Vargas et al. 2010) and maintains the circadian rhythmicity of sensitivity to sex pheromones in moths (Linn and Roelofs 1986; Linn et al. 1994).

The distribution of the investigated transmitters and neuropeptides did not differ between the species nor between males and females. Differences in physiological and behavioral responses to odors may be reflected by the quantitative distribution of OSNs in the antenna (as shown in this study), whereas the distribution of neuroactive substances in the AL may reflect common mechanisms for processing relevant information. However, only minor alterations in AL wiring of excitatory and inhibitory circuits may dramatically change the perception of plant odors as was recently suggested in a theoretical model (Cunningham 2012). Cunningham suggested that specific interneuron algorithms categorize an odor as either host or nonhost. An adaptive change in the algorithm could easily alter the categorization so that a former nonhost is now perceived as a host (or the other way around) using existing input channels. Hence, we cannot exclude that differences in AL wiring, not detectable at the resolution used in this study, contribute to the species-specific discrimination abilities.

Output neurons from the AL were found, not surprisingly, to innervate particularly 2 protocerebral areas, the calyces of the mushroom bodies and the lateral protocerebrum (LP). We could identify 3 different ACTs. One tract projects directly to the LP in a lateral path, thus identical to the outer ACT in moths (Anton and Homberg 1999). The remaining 2 tracts leave the AL through a common medial bundle. However, the tracts split and one medial tract commence to the calyx of the mushroom bodies before finally reaching the LP. The other part makes a sharp lateral turn and targets the LP. The 2 latter tracts are similar to the medial and inner ACT found in moths (Anton and Homberg 1999). In other insect orders, the tracts may look different (Galizia and Rössler 2010), but the architecture of AL output tracts seems to be evolutionary conserved within Lepidoptera.

In summary, we performed an initial study of the anatomy and distribution of neuroactive substances of the butterfly olfactory system. In many respects, the olfactory system

resembles that of moths, however, somewhat numerically reduced. Between the 2 species the number of olfactory sensilla differed, otherwise the systems were remarkably similar. Due to the recent interest in butterfly olfaction, this anatomical study will hopefully be a springboard for future physiological and in-depth neuropharmacological studies.

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