

Octopaminergic innervation and a neurohaemal release site in the antennal heart of the locust *Schistocerca gregaria*

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Abstract A detailed account is given by the octopaminergic innervation of the antennal heart in *Schistocerca gregaria* using various immunohistochemical methods. Anterograde axonal filling illustrates the unilateral innervation on the medial ventral surface of the pumping muscle of the antennal heart via the paired corpora cardiaca nerve III. In addition, antibody staining revealed that ascending axons of this nerve terminate at the ampullae of the antennal heart forming synaptoid structures and extensive neurohaemal release sites. Due to the innervation by two dorsal unpaired median neurons, the presence of the biogenic amines octopamine and tyramine could be visualized by immunocytochemistry in an insect antennal heart for the first time. The data suggest that tyramine acts as a precursor and not purely as an independent transmitter. While the octopaminergic fibers innervating the pumping muscle of the antennal heart indicate a cardioregulatory role, we conclude that octopamine released from the neurohaemal area is pumped into the antennae and an involvement in the modulation of the antennal sensory sensitivity is discussed.

Keywords Octopamine · Tyramine · Accessory pulsatile organ · Circulation · Sensory modulation

Introduction

Insects possess accessory pulsatile organs to facilitate haemolymph circulation through long body appendages (reviews: Pass 1998, 2000; Pass et al. 2006; Wirkner et al. 2013). These auxiliary hearts are autonomous pumps, which work independently from the dorsal vessel, the primary pumping organ of the insect circulatory system. Accessory pulsatile organs, which supply haemolymph to the antennae, were initially discovered in cockroaches by Pawlowa (1895) and later described for numerous other insects (review: Pass et al. 2006). The most detailed analysis of the functional anatomy and physiology of an antennal heart is that for the cockroach, *Periplaneta americana* (Hertel et al. 1985, 1988, 2012; Pass 1985; Pass et al. 1988a, b; Hertel and Penzlin 1992; Richter and Hertel 1997).

Likewise, antennal heart innervation was first investigated in detail in *P. americana* (Pass et al. 1988a). Retrograde cobalt fills of the antennal heart nerve showed that the somata of the innervating neurons are located in the suboesophageal ganglion (SOG). These neurons include dorsal unpaired median (DUM) neurons and a pair of contralateral neurons whose axons run together via an anterior branch of the paired *nervus corporis cardiaci III* (NCC III) into the corpora cardiaca and from there to the antennal heart. Numerous peripheral branches of this nerve ramify at the surface of the pumping muscle, and a large number of axon terminals are found within the ampulla wall. Transmission electron microscopy revealed that these terminals contain neurosecretory granules indicating that the antennal heart has a neurohaemal function, in addition to that of a

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circulatory motor (Beattie 1976; Pass et al. 1988a). Studies in imagines of *Locusta migratoria* (Bräunig 1990) revealed almost the same innervation pattern of the antennal heart as in the cockroach. It is innervated by a branch of the NCCIII which contains the axons of two DUM neurons and a pair of contralateral motor neurons (CN3) located in the suboesophageal ganglion.

DUM neurons are present in insects in all segmental ganglia of the ventral nerve cord (reviews: Stevenson and Spörhase-Eichmann 1995; Bräunig and Pflüger 2001). They possess somata in the dorsal area of these ganglia in a medial position and have bifurcating axons projecting bilaterally to the periphery (Duch et al. 1999; Watson 1984). Earlier biochemical or immunohistochemical studies suggest that all DUM neurons contain octopamine (OA; Crossman et al. 1971; Duch et al. 1999; Evans 1985; Hoyle 1975; Orchard and Lange 1985; Kononenko et al. 2009). The enzyme tyramine- β -hydroxylase (T β H) converts tyramine to OA through the addition of a hydroxyl group in β -position, which makes tyramine the biological precursor of OA (Roeder 2005). OA is recognized as one of the major modulators within the nervous system of invertebrates and is from its chemical structure and biological function related to noradrenaline in vertebrates (reviews: Roeder 1999; Verlinden et al. 2010). Centrally applied OA can elicit rhythmic behaviors in locusts, such as flight, running, and swimming (Ramirez and Pearson 1991; Sombati and Hoyle 1984; Stevenson and Kutsch 1986; Rillich et al. 2013). In addition, it modulates visceral muscle contractions of the female reproductive tract of locusts and cockroaches (review: Lange 2009). In particular, it was shown that OA decreases the frequency and amplitude of myogenic contractions in oviducts (Lange and Orchard 1986; Orchard and Lange 1985, 1986, 1987; Kalogianni and Pflüger 1992) and spermathecae (Clark and Lange 2003). Furthermore, a modulatory effect of OA has been reported for insect circulatory organs (Papaefthimiou and Theophilidis 2011; reviews: Miller 1985; Neckameyer and Leal 2009).

The presence of OA in the antennal heart of *P. americana* could be demonstrated by a radioenzymatic assay (Pass et al. 1988b). The measured OA concentrations are with 9500 pg mg⁻¹ wet mass in male antennal hearts extraordinarily high compared to other insect tissues (see tables in Evans 1985; Roeder 1999). Remarkably, more than 90% of the OA is concentrated in the outermost lateral part of this organ consisting primarily of the ampullae. This strongly indicates that the bulk of OA in the antennal heart is localized in the neurohaemal area associated with the ampullae. All hormones and other substances released from this site into the haemolymph are further pumped into the antennae. Since the circulation time within the antennae is considerable, it is evident that the target of quickly degraded hormones, such as OA (Goosey and Candy 1982), must be

located within the antennae. In ectognathan insects, only the basal segment, the scape, contains muscles (Imms 1939) which are innervated aside from several motor neurons also by DUM neurons of the SOG (Honegger et al. 1990). Most of the antenna length is, however, formed by the flagellum that, aside from the extensive sensory apparatus, contains only epidermis and a few tracheae. In view of this fact, it appears most likely that the sensory receptors in the flagellum are the target of hormones released from the antennal heart.

The antennal sensory apparatus comprises a multitude of sensory organs for various modalities, although olfactory sensilla are prevailing (reviews: Schneider 1964; Keil 1999). Olfactory guided behavior exhibits a great plasticity, and a large number of investigations show that insects modulate their olfactory system according to their physiological state upon interaction with their environment (review: Dukas 2008; Gadenne et al. 2016). In particular, OA has proven to be involved as a modulator that enhances the responsiveness to olfactory and other sensory stimuli in insects (Mercer and Menzel 1982; Linn and Roelofs 1986; Linn et al. 1992, 1996; Matheson 1997; Pophof 2000; Grosmaître et al. 2001; Flecke and Stengl 2009; Jung et al. 2013; Hillier and Kavanagh 2015; review: Farooqui 2007). Any of the various levels of the olfactory pathway are possible targets of the modulatory interference by OA, including peripheral receptors, as well as the central processing centers in the brain (reviews: Hansson and Christensen 1999; Stengl 2010; Galizia 2014; Gadenne et al. 2016).

Recently, tyramine (TA) was suggested to act as an individual transmitter with a possible antagonistic function to OA (Downer et al. 1993; Fox et al. 2006; Fussnecker et al. 2006; Lange 2009; Roeder 2005). In *Drosophila melanogaster*, it was shown that TA antagonizes the effect of OA at the neuromuscular junctions (Nagaya et al. 2002) and functions synergistically on oviducts and the spermatheca in locusts (da Silva and Lange 2008; Donini and Lange 2004). Furthermore, Kononenko et al. (2009) showed that purely tyramineric cells exist in the locust brain, SOG, and abdominal fused ganglia.

The aim of the present study was to investigate for the first time the extent of the innervation and the distribution of varicosities and synapses in the antennal heart of *Schistocerca gregaria* by several neuronal and synaptic markers. We used synapsin to label areas with high synaptic density (see Kurylas et al. 2008; Michels et al. 2005) and to indicate synaptic release sites (see Benfenati et al. 1989). The neuroanatomical tracer neurobiotin was applied in anterograde tracing and, thus, synaptic structures and varicosities could be visualized (see Heinrich et al. 1998). Neurobiotin, together with synapsin, enabled the visualization of axonal varicosities and likely presynaptic release sites. Furthermore, the octopaminergic and tyramineric innervation of

the antennal heart of *S. gregaria* was visualized by applying respective antibodies.

Materials and methods

Animals

For the present study, antennal hearts of both sexes of *S. gregaria* were dissected and examined. The locust specimens were taken from our crowded laboratory colony that is maintained under a constant light regime (12:12/h—light:dark) at appr. 30 °C, and with a relative humidity of 40–60%.

All experiments adhere to the Principles of Laboratory Animal Care and the German Law on the Protection of Animals (Deutsches Tierschutzgesetz).

Dissection of *S. gregaria*

Prior to the experiments, the animals were stored overnight in a refrigerator at approximately 4 °C to slow metabolism, in particular, neuronal activities. Heads were separated and prefixed in the respective fixative solution for 90 min. To facilitate dissection of the antennal hearts, the heads were stabilized in the preparation dish by melted beeswax. The location of the antennal heart can be easily recognized from the exterior by a slight bulge of the cuticle close to the medial margin of each antennal base (Fig. 1a). To prepare an antennal heart for the labeling experiments, a rectangular-shaped window was cut between the two antennae. Once the antennal heart dilator muscle was located, the surrounding

tracheae and fatty tissue were carefully removed and the antennal heart was dissected out of the head capsule together with parts of the frontal cuticle and then pinned to a small Sylgard dish. To avoid damage to the antennal heart, the remaining fatty tissue around it was initially left undisturbed. After fixation with the respective fixative solution, the preparations were cleaned of the remaining fatty tissue and tracheae during the ascending dehydration series with ethanol (50, 70, 90, and 100%).

Semi-thin sections

To produce serial semi-thin sections, the heads were fixed in a formaldehyde–acetic acid–ethanol mixture (FAE, see Beutel et al. 2014). After dehydration in an ascending acetone series, the objects were embedded under vacuum impregnation in Agar low viscosity resin (Agar Scientific). Following the procedure of Blumer et al. (2002), serial semi-thin sections (thickness 1 µm) were cut with a Leica EM UC6 microtome using a Histo Jumbo Diatome diamond knife. The sections were stained with a mixture of 1% azure and 1% methylene blue in a 1% aqueous borax solution, diluted 1:20 with distilled water, for approximately 30 s at 60–70 °C.

Anterograde nerve staining with neurobiotin

Anterograde staining (axonal fill) of the circumoesophageal connective was conducted to elucidate the innervation of the antennal heart via the NCC III. First, the locusts were stored in a refrigerator for 30–60 min. Afterward, the heads were severed posterior to the compound eyes and stabilized with

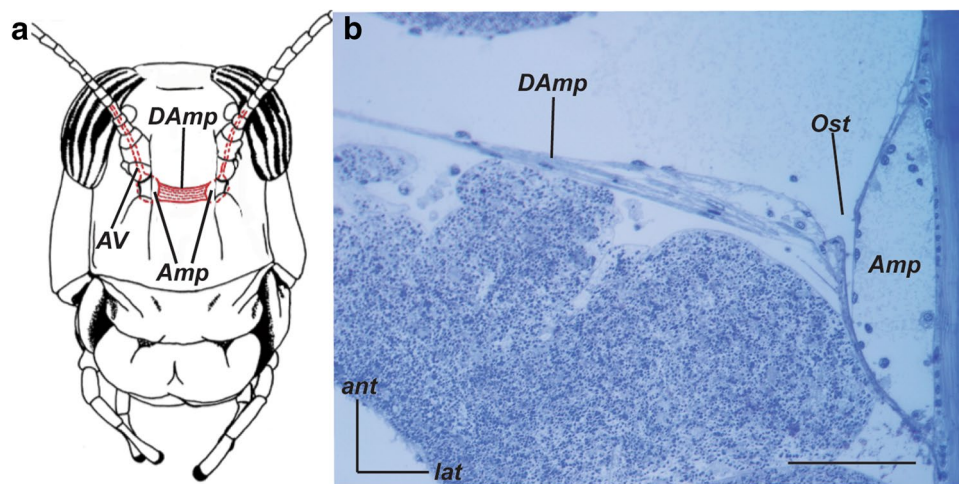


Fig. 1 Anatomy of the antennal heart in *S. gregaria*. **a** Frontal view of a locust head with the antennal heart drawn in by red lines. An ampulla is located at the base of each antenna to which a long antennal vessel is connected; the two ampullae are joined by an ampulla dilator muscle. **b** Horizontal semi-thin section through right half of

the antennal heart. The ampulla is attached to the frontal cuticle with the dilator muscle inserting at the inner ampulla wall. Slit-like valved ostium located at the frontal side of the ampulla, scale bar 100 µm. *ant* anterior, *lat* lateral, *DAmP* dilator of ampulla, *Ost* ostium, *Amp* ampulla, *AV* antennal vessel

minute pins in Sylgard dishes. After carefully removing fatty tissue and tracheae, the circumoesophageal connectives were severed just anterior to the SOG and the anterior end of one connective was embedded in a vaseline pool first filled with distilled water to osmotically widen the axons and to test for leakiness of the vaseline pool. After 10 min, the distilled water was removed and replaced with a 3% neurobiotin solution (Neurobiotin tracer, Vector Laboratories). The pool was sealed airtight and the preparation placed in a moist chamber. The chamber was stored in a cool room (approximately 4 °C) for 2–4 days to enable diffusion of the neurobiotin dye throughout the nerves.

Neurobiotin/streptavidin–fluorochrome labelling and immunohistochemistry on whole-mount dissections

To use neurobiotin/streptavidin and synapsin as labels, the dissections of the antennal heart were fixed in a 4% paraformaldehyde fixative solution (PFA) for 1 h and then washed in 0.1 M phosphate buffered saline (PBS) for 3 × 10 min. The preparations were dehydrated in an ascending ethanol series (50, 70, 90, and 100%) for 10 min each and then degreased in xylene for 2 × 5 min. Subsequently, they were rehydrated in a descending ethanol series (100, 90, 70, 50%; each 10 min) and then washed in 0.1 M PBS for 1 × 15 min. Following this procedure, the dissections were washed in 1% Triton X-100 in 0.1 M PBS (PBS-TX) for 4 × 15 min and preincubation was conducted for 1 h in a solution containing 10% normal goat serum (NGS) in PBS-TX.

Neurobiotin/streptavidin labeling

The dissected antennal hearts, prepared according to the aforementioned procedure, were incubated with Cy2 or Cy5 conjugated biotin-binding protein Streptavidin (dissolved 1:100 in PBS-TX) for 1 day at room temperature. After 24 h, the preparations were washed in 0.1 M PBS for 3 × 10 min and dehydrated in an ascending ethanol series (50, 70, 90, and 100%) for 10 min each. They were permeabilized in a 1:1 mixture of 100% ethanol and methyl salicylate for 10 min and then transferred into pure methyl salicylate (for detailed protocol, see online resource 1).

Synapsin labeling

To visualize synaptic terminals, we used an established primary antibody, anti-Synorff1 (monoclonal anti-mouse, DSHB, Würzburg, Germany), that recognizes the vesicular protein synapsin. The marker was dissolved 1:10 in a prepared buffer solution, containing PBS-TX, 1% NGS, and 1% sodium azide (NaN₃). After storing the incubating dissections in a cool room (approximately 4 °C) for 4–5 days,

they were washed in PBS-TX for 6 × 20 min. The dissections were incubated in a Cy2 or Cy5 conjugated antibody (goat-anti-mouse; 1:100) for 1 day at room temperature. The following day, the preparations were washed in 0.1 M PBS for 3 × 20 min and dehydrated with an ascending ethanol series (50, 70, 90, and 100%, each for 10 min). They were permeabilized in a 1:1 mixture of 100% ethanol and methyl salicylate for 10 min and then transferred into pure methyl salicylate (for detailed protocol, see online resource 2).

Octopamine and tyramine labeling

To label TA and OA, the dissected antennal hearts were fixed in a picric acid fixative mixture [6.25% glutaraldehyde, 75% picric acid, 5% (glacial) acetic acid, and 1% sodium metabisulfite (SMBS)] for 1 h. Then, they were washed for 2 × 10 min in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6) and kept in the same buffer solution overnight. The following day, they were washed again for 10 min with the above-mentioned solution, dehydrated in an ascending ethanol series (50, 70, 90, 100%; each for 10 min), and degreased in xylene for 2 × 5 min. The dissections were dehydrated in a descending ethanol series (100, 90, 70, 50; each 10 min) and then washed in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6) for 15 min. To break up the glutaraldehyde bonds, they were washed in 1% sodium borohydride (0.04 g sodium borohydride in 4 ml Tris–HCl) for 10 min and then 15 min in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6). The dissections were washed in 1% Triton X-100 in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6) to increase permeability and subsequently treated with 10% NGS in 1% Triton X-100 in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6) for 1 h. Following preincubation, incubation with primary antibodies was applied following largely the procedures developed for these two antibodies by Kononenko et al. (2009). The primary anti-tyramine antibody (polyclonal anti-rabbit, Chemicon, Temecula, CA, USA) was dissolved 1:200 in a prepared buffer solution, containing 1% Triton X-100 in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6), 1% NGS, and 1% sodium azide (NaN₃). The primary anti-OA antibody (monoclonal mouse anti-OA, Jena, Bioscience) was dissolved 1:1000 in the same buffer solution. After storing the incubating dissections in a cool room (approximately 4 °C) for 4–5 days, they were washed 6 × 20 min in 1% Triton X-100 in 0.1 M Tris–HCl–SMBS (0.45%, pH 7.6). Thereafter, the dissections were incubated in Cy2- or Cy5-conjugated antibodies [goat-anti-rabbit for anti-tyramine (conjugated to Cy2) and goat-anti-mouse for anti-OA (conjugated to Cy5), both 1:100] for 1 day at room temperature. The following day, the preparations were washed in 0.1 M Tris–HCl for 4 × 20 min and subsequently dehydrated with an ascending ethanol series (50, 70, 90, and 100%, each for 10 min). The specimens were permeabilized in a 1:1 mixture of 100%

ethanol and methyl salicylate for 10 min and then transferred into pure methyl salicylate (for detailed protocol see online resource 3).

Image acquisition

The staining of the antennal hearts was initially assessed using a fluorescence microscope (Zeiss Photomicroscope Axiophot). More than 120 antennal hearts treated with different staining methods were evaluated in this way, and preparations with the most intensive staining were used for further processing by confocal scanning microscopes (TCS SP2, Leica, Germany; TCS SP8, Leica, Germany). Different objectives such as HC PL 5×/0.15, HC PL 10×/0.5, and HC PL APO CS2 20×/0.7 mm were used for scanning. The optical zoom factor varied from 1 to 1.5 for an overview and from 2.5 to 6 for a detailed scan. Each antennal heart was scanned with a resolution of 2048 × 2048 pixels. Depending on the thickness of the preparation, the appropriate slice numbers of the serial stacks were scanned with step sizes of 1.04 μm and an average from 2 to 3 times. Two different lasers, an Argon/Krypton laser at 488 nm and a Helium/Neon laser at 633 nm, were used to scan the images simultaneously. Images were analyzed using the open source software ImageJ 1.48r (Java 1.6.0_65, National Institutes of Health, USA). For detailed image acquisition, see online resource 4.

Results

Anatomy of the antennal heart

The functional anatomy of the antennal heart in *S. gregaria* resembles that described for other species of locust (Bayer

1968; Pass 1991). It consists of pulsatile ampullae, which are oval-shaped vesicles that are attached to the frontal cuticle medially of each antenna base (Fig. 1). Each ampulla is connected to a vessel, which extends into the antenna up to the apex. The two ampullae are linked by a transverse muscle, which inserts at their inner walls. Contraction of this muscle results in a simultaneous diastole of both ampullae, and haemolymph enters through slit-like valved ostia (Fig. 1b). The systole phase follows when the transverse dilator muscle relaxes, obviously due to the elasticity of the ampulla walls (see Pass 1985). Haemolymph is thus forced through the antennal vessel up to the apex where it emanates through a terminal pore into the antennal haemocoel where it flows in the opposite direction back to the head capsule. Therefore, there is a clearly a directed haemolymph flow through the antennae. The dimensions of the transverse dilator muscle are: 1350 μm (mean ± SD 162 μm, $n = 20$) in length, 175 ± 43 μm width, with a mean diameter of 26 ± 9 μm.

Ramification of NCC III terminals

Axonal tracing with neurobiotin

The dilator muscle of the ampullae receives innervation from four neurons (two DUMs and a pair of motor neurons CN3) located in the SOG. Among them are DUM neurons, whose axons run through the circumoesophageal connectives and the nerve NCC III, which extends to the corpora cardiaca and from there to the antennal heart (Bräuning 1990). In the present study, the connectives of all specimens were unilaterally stained by neurobiotin to determine whether the antennal heart receives lateral innervation from each NCC III nerve. Figure 2 shows a clearly labeled octopaminergic fiber that innervates the antennal heart via the medial ventral surface (Fig. 2b, dashed white circle) and separates

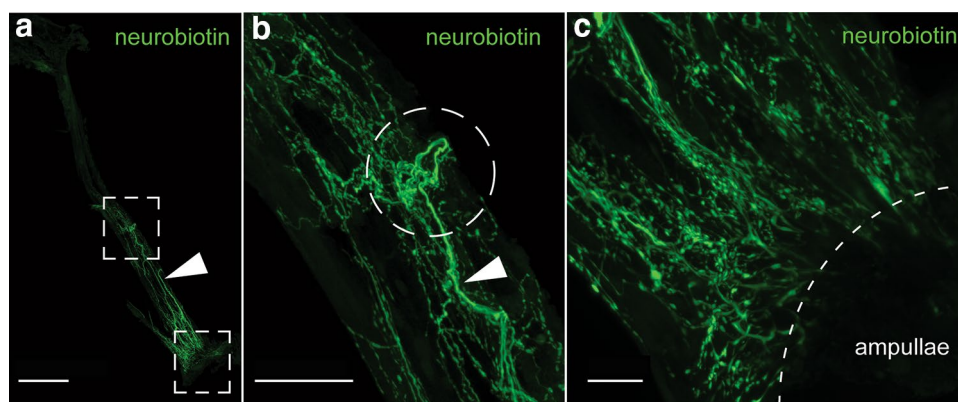


Fig. 2 Labeled fibers on the antennal heart as a result of anterograde staining of one circumoesophageal connective with antibodies against neurobiotin/streptavidin. **a** Neurobiotin-labeled structures and varicosities run towards the ampulla (white arrow), scale bar 200 μm. **b**

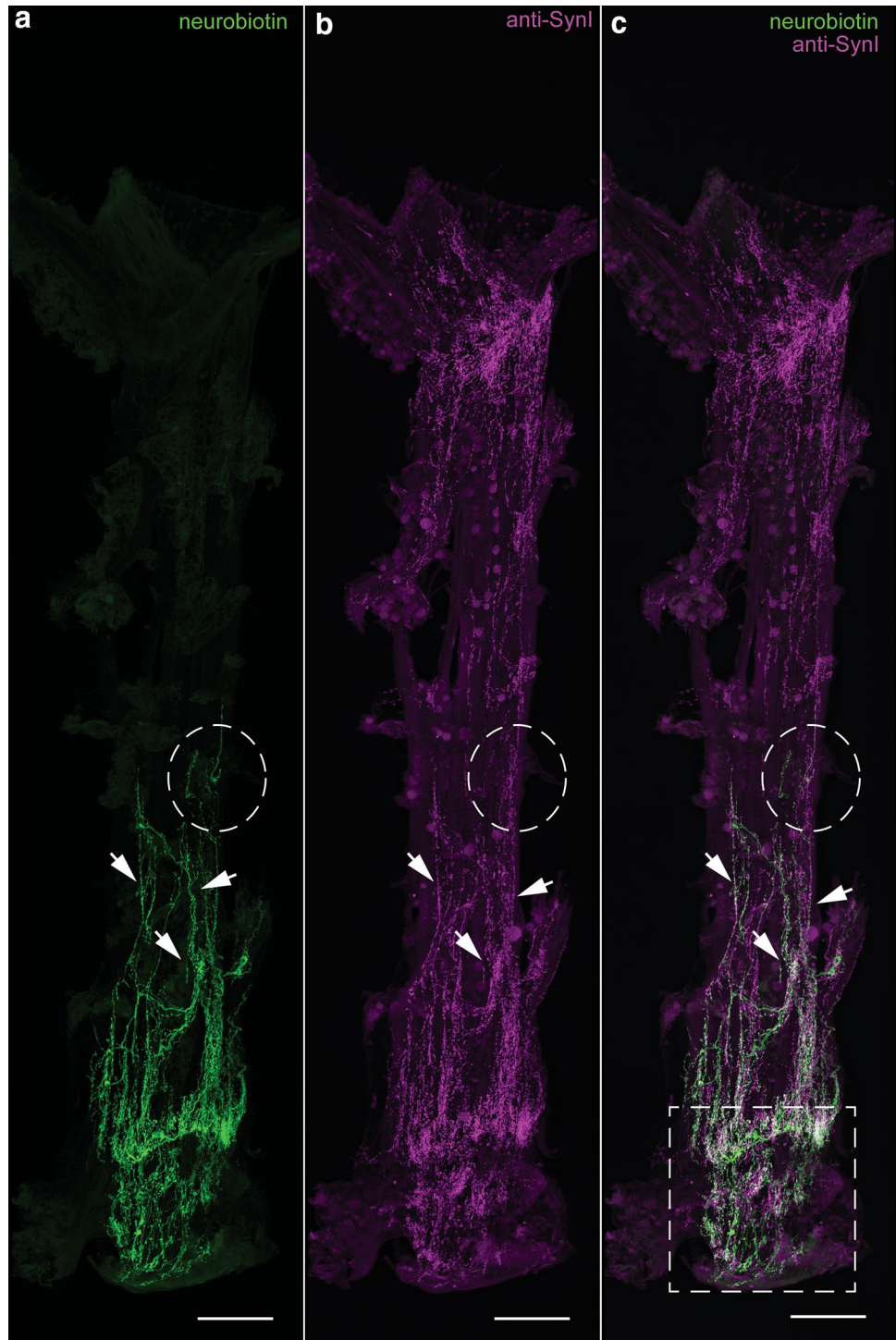
Enlarged section of the neurobiotin-labeled varicosities. The axon collaterals (white arrow) and nerve entrance (dashed white circle) are visible, scale bar 50 μm. **c** Neurobiotin-labeled beaded fibers and varicosities near the ampulla wall (dashed line), scale bar 20 μm

unilaterally into collaterals, which run towards the ampulla (Fig. 2, white arrows).

At the point where nerve NCC III joins the dilator muscle, some minor beaded fibers continue for a short distance in the contralateral direction, whereas all main collaterals appearing as beaded fibers of various thicknesses extend in the opposite direction towards the ampulla where they form

a dense, plexus-like network of terminal fibers. Especially at the wall of the ampulla, vast amounts of beaded fibers and thick profiles are visible (see Figs. 2c, 3).

Fig. 3 Immunoreactive varicosities and beaded fibers on the antennal heart stained with antibodies against neurobiotin/streptavidin and synapsin. **a** Neurobiotin labeling reveals the nerve entrance (*dashed white circle*), axon collaterals, and ramifications (*white arrows*). **b** The same stack of images with anti-synapsin staining of neuronal structures reveals the same nerve entrance (*dashed white circle*) and axon collaterals (*white arrows*). **c** Double labeling of neurobiotin (*green*) and anti-synapsin (*magenta*) shows co-localization of the nerve entrance (*dashed white circle*), beaded fibers, and axon collaterals (*white arrows*), *scale bar* 100 μm (see Fig. 4 for *dashed square*)



Neurobiotin/streptavidin and synapsin labeled structures

Synaptic zones were visualized using anti-synapsin simultaneously with neurobiotin to determine presynaptic release sites. The preparations show numerous active zones near the ampulla wall and in the varicosities of the beaded fibers on the antennal heart (Fig. 3). Neurobiotin reveals axonal structures, which ramify from the stained nerve NCC III, and synapsin illustrates presynaptic sites and, thus most likely, the locations of vesicle release sites such as in neuromuscular junctions or neurohaemal terminals.

The innervation pattern shown in Fig. 3a once again reveals the nerve supplying the antennal heart on the medial ventral surface (Fig. 3a, white circle) and the ramifications of one labeled fiber into axon collaterals (Fig. 3, white arrows). The synapsin staining reveals synaptic sites, which are clearly located on beaded fibers and mainly label varicosities proceeding towards the ampullae. The ramifications of the axon collaterals are more widely distributed in the median section of the muscle and become particularly dense near the wall of the ampulla (Fig. 3a–c). Double labeling of synapsin and neurobiotin (Fig. 3c) shows the varicosities of the beaded fibers stained by neurobiotin co-localizing with the synapsin immunoreactivity.

The enlarged section of the ampulla (dashed square in Fig. 3c) reveals a closer aspect of the dense area mentioned above (Fig. 4). The co-localizations are visible in Fig. 4bi–iii (white arrows). The synaptic active zones appeared as loosely arranged boutons of a “chain” (beaded fibers) in all preparations.

Allocation of octopaminergic and tyraminergetic structures

To reveal a possible co-localization of labeled neuromodulatory structures with TA/OA, a polyclonal antibody against TA was applied simultaneously to anti-OA.

Figure 5ai displays the pattern of OA immunoreactivity on the antennal heart and near the ampulla wall. Each half of this visceral muscle receives separate innervation and both ends located towards the ampulla were densely covered by neuromodulatory varicosities and structures. Anti-tyramine staining reveals similar structures (Fig. 5aii). Again, these varicosities are densely distributed near the ampulla wall, similar to the octopaminergic structures. Figure 5aiii demonstrates that tyramine immunoreactivity on the antennal muscle is exclusively limited to neuromodulatory structures also labeled by anti-OA. This co-localization is clearly visible, especially close to the ampulla wall. The double-labeled octopaminergic and tyraminergetic structures appear as chains

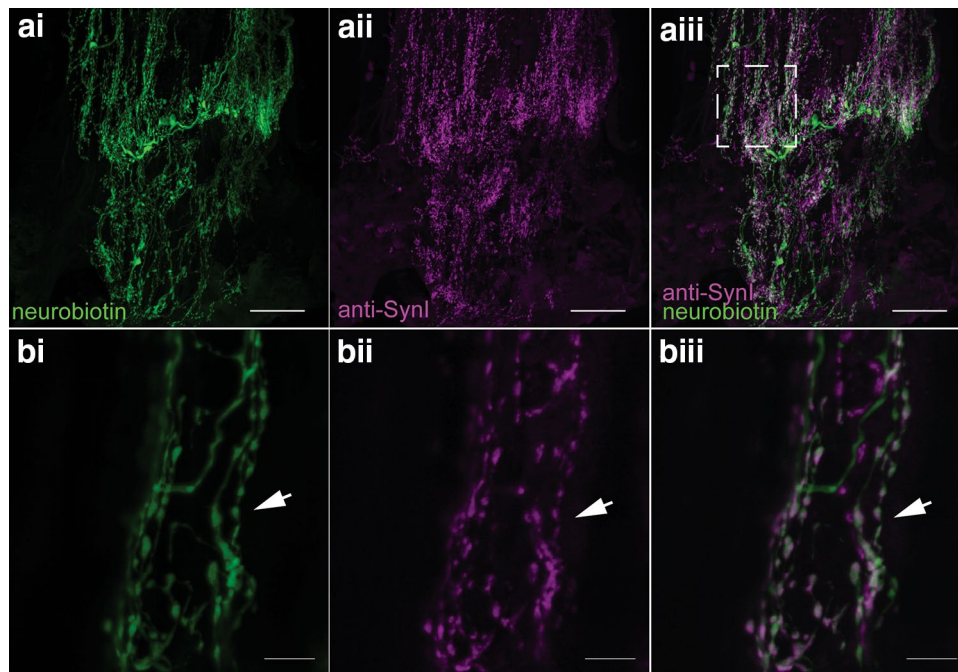
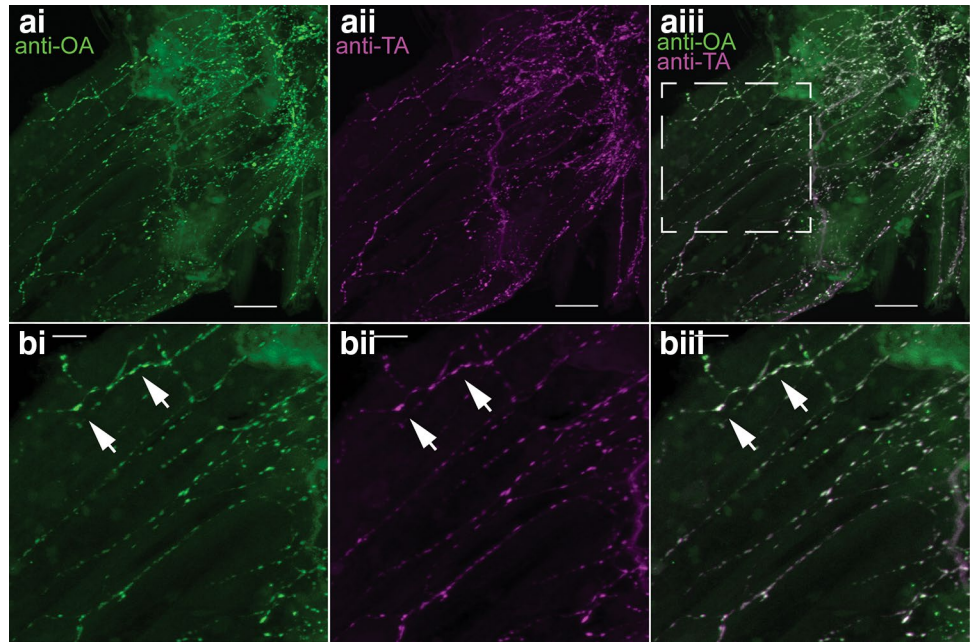


Fig. 4 Enlarged view of the dense area near the ampulla wall (dashed square in c) **a** Neurobiotin-labeled beaded fibers and varicosities near the ampulla wall (**ai**). Anti-synapsin staining reveals a strong labeling of active zones (**aii**). Double labeling with neurobiotin (green) and anti-synapsin (magenta) reveals a co-localization of a vast amount of fibers and varicosities (**aiii**), scale bar 50 μ m. **b**

Enlarged view of dashed square in Fig. 5aiii. A group of neurobiotin-labeled beaded fibers (**bi**, white arrow) are revealed. The labeling of anti-synapsin shows the same group of beaded fibers (**bii**, white arrow). Double labeling of neurobiotin (green) and anti-synapsin (magenta) shows co-localizations of the before mentioned group of beaded fibers (**biii**, white arrow), scale bar 10 μ m

Fig. 5 Octopaminergic and tyraminergetic fibers appear mostly in immediate vicinity of the ampullae and are co-labeled on the antennal heart muscle. **a** Octopamine immunoreactivity reveals the distribution of varicosities on the muscle and near the ampullae (**ai**). The same stack of images reveals tyraminergetic fibers with the similar distribution (**aii**). The co-localization of octopamine and tyramine is shown in chains of loosely arranged varicosities on the antennal heart muscle (**aiii**), scale bar 50 μm . **b** Detailed view of octopaminergic (**bi**) and tyraminergetic (**bii**) fibers (dashed square in **aiii**). Co-localized fibers of tyramine and octopamine staining (**biii**, white arrows), scale bar 20 μm



of loosely arranged varicosities and, thus, each contains both transmitters (white arrows in Fig. 5bi–iii).

Co-localization of anti-OA and anti-tyramine is presented for a detailed part of the visceral muscle (Fig. 5bi–iii). Figure 5ai, bi reveals the parts of beaded fibers labeled by anti-OA. Anti-tyramine staining reveals a similar pattern of varicosities (Fig. 5aai, bii).

Discussion

In this study, we present data showing the extent of innervation at the antennal heart in *S. gregaria*. For the first time in insects, the distribution of the synapses, varicosities, and neurohaemal terminal on this organ was visualized using several neuronal and synaptic markers. With respect to the biological role of the octopaminergic fibers innervating the antennal heart, we must differentiate between (1) the innervation of the dilator muscle and (2) the supply of the neurohaemal release sites in the ampulla area of this organ.

Nerve branching, synapses, and neurohaemal release sites

The previous studies in *P. americana* (Pass et al. 1988a) and *L. migratoria* (Bräunig 1990) revealed that the neurons that are associated with their antennal hearts are located in the SOG. Axons of contralateral neurons and DUM neurons located there run via the paired NCC III into the CC and further to the antennal heart. Since the innervation proceeds unilaterally via the circumoesophageal connectives (Bräunig 1990; Pass et al. 1988a), only one connective was

stained with neurobiotin to visualize the lateral innervation of this visceral muscle. This tracer distributes quickly through nerves and has proved to be best suited for axonal tracing (Heinrich et al. 1998). The staining revealed specific innervation via the medial ventral surface. Axon collaterals of the NCC III axon ramify towards the ampulla, where the axons develop into a dense meshwork of arborizations and then terminate at the ampulla wall.

Another staining technique comprises simultaneous anti-synapsin labeling. It is well known that synapsin labels synaptic release sites and active zones (Benfenati et al. 1989). Simultaneous staining with anti-synapsin and neurobiotin shows a similar density of arborizations near the ampulla and confirms the lateral innervation. It also shows a major overlap in the region of the ampulla, which indicates synaptic release sites. This result of *S. gregaria* reiterates the condition in *P. americana* where numerous neurosecretory terminals form a neurohaemal area (Beattie 1976; Pass et al. 1988a).

Octopamine and tyramine immunoreactivity

Since all efferent DUM neurons biochemically and immunohistochemically examined contain OA (cf. Evans 1985; Orchard and Lange 1985, Stevenson and Spörhase-Eichmann 1995; Kononenko et al. 2009), this biogenic amine was suspected to occur in the nerve fibers associated with the antennal heart. In accordance with earlier studies that used a radioenzymatic assay and that demonstrated a high content of OA in the antennal heart of the cockroach, especially in the region of the ampullae (Pass et al. 1988b), both

octopaminergic and tyraminergetic fibers were shown in *S. gregaria* to be highly prominent near the area of the ampulla (Fig. 5).

It is possible that TA acts only as a precursor for OA, because we have no evidence, suggesting that OA's precursor tyramine could act as an independent transmitter in this system. The immunoreactivity provides further evidence that the described innervation of the antennal heart belongs to the SOG DUM neurons. In addition, the occurrence and prominent distribution of loosely arranged boutons near the ampulla resembled in some aspect that of type II synapses described for vertebrates (e.g. Hassler and Chung 1976) and of type II terminals described for insect muscle in *D. melanogaster* (Atwood et al. 1993). The tyraminergetic beaded fibers proved to be exclusively limited to the octopaminergic fibers, which leads us to conclude that tyramine is not an independent transmitter in the antennal heart. In addition, the previous studies also identified varicosities with a colocalization of TA and OA in skeletal muscles (Kononenko et al. 2009; Stocker 2011). Tyraminergetic innervation has also been shown in visceral muscles (da Silva and Lange 2008; Donini and Lange 2004). Orchard and Lange (1985) examined the functionality of TA and in connection with the results of the latter study, they showed that a purely tyraminergetic innervation of visceral muscle is not present. As far as known, TA exclusively binds to octopaminergic receptors in muscle, and it is unlikely that TA acts as an independent neuromodulator in the antennal heart, as Roeder (2005) and Kononenko et al. (2009) showed for the central nervous system in insects. Studies investigating TA at the spermatheca of *L. migratoria* showed that both transmitters act on OA receptors (da Silva and Lange 2008).

Cardiomodulatory function of octopamine

The physiology of antennal hearts has been investigated so far only in cockroaches (Hertel et al. 1985, 1988; Hertel and Richter 1997; Hertel and Penzlin 1992; Lange et al. 1993; Richter and Hertel 1997; Predel et al. 2004) and mosquitoes (Boppana and Hillyer 2014; Suggs et al. 2016). In *Periplaneta*, it was shown that contractions of this circulatory organ are based on a myogenic automatism, while frequency and amplitude of the muscle contractions are regulated by the antennal heart nerve (Hertel et al. 1988). In most cases, OA applied to isolated antennal hearts causes a short transient cardiac block followed by a decreased beating frequency (Hertel et al. 1988; Hertel and Penzlin 1992).

OA also affects the pumping frequency of the primary circulatory pump of insects, the dorsal vessel ("heart"), although the results achieved from different species provide no clear picture (Zornik et al. 1999; Tsai et al. 2004; Papaefthimiou and Theophilidis 2011; reviews: Miller 1985; Neckameyer and Leal 2009). This could be due to

different species-specific effects, and eventually also to biphasic effects at various concentration levels as described by Papaefthimiou and Theophilidis (2011). In *L. migratoria*, rhythmically active DUM neurons innervate the dorsal vessel (Ferber and Pflüger 1990, 1992). These two so-called "DUM-heart neurons" were studied in great detail, and a co-localization of OA (Stevenson and Pflüger 1994) together with three other transmitters was determined (RFamide-like peptide: Ferber and Pflüger 1992; taurine: Stevenson 1999; nitric oxide: Bullerjahn et al. 2006).

Earlier studies on the innervation of visceral muscles, for example the oviduct muscle of *L. migratoria*, investigated both the release of OA and the neuropeptide proctolin (Orchard and Lange 1987). It turned out that the release of both transmitters was frequency-dependent, with the maximum release of OA at 5 Hz and maximum release of proctolin at 30 Hz. This strengthened the argument that OA is one of the natural regulators of insect visceral muscles and that it modulates muscle contractions (Orchard and Lange 1985, 1986). In the antennal heart of *Periplaneta*, the presence of proctolin has been demonstrated by immunohistochemistry, high-performance liquid chromatography, and mass spectrometry (Hertel et al. 1995, 2012; Predel 2001). Moreover, proctolin was shown to have a strong excitatory effect on the beating frequency of this circulatory pump (Lange et al. 1993; Hertel et al. 1995). In addition, a strong allatostatin immunoreactivity has been demonstrated in nerve fibers innervating the antennal heart of the cockroach *Diploptera punctata* (Woodring et al. 1992).

In summary, for at least the cockroach and the locust, it was ascertained that DUM neurons are involved in the innervation of the antennal heart, as well as the dorsal vessel. OA is suggested to act as a modulator of their beating frequency. A concerted action together with proctolin and other neuropeptides is probable.

Octopamine in the neurohaemal area of the antennal heart

Of particular interest is the well-developed neurohaemal release site in the ampulla region of the antennal heart. The anatomical condition in *Schistocerca* closely resembles that in *Periplaneta* (see: Fig. 15 in Pass et al. 1988a). In the cockroach, the determination of OA was performed with a radioenzymatic assay, which permitted only an indirect conclusion that it is localized in the neurosecretory terminals of the ampullae (Pass et al. 1988b). The present results in *Schistocerca* now clearly demonstrate that there are octopaminergic terminals in the region of the ampullae. Therefore, we can assume that OA is released there and pumped into the antennae. In *Schistocerca*, the cuticle of the antennae is non-transparent, and therefore, the circulation time cannot be calculated by observation of the haemocyte flow.

However, in *Periplaneta*, it was possible to determine the velocity of haemocyte movement by visual observation, which led to a rough estimate of 10 min for a full circulation cycle through the antenna (Pass et al. 1988b). This time span corresponds approximately to the half-life of OA in insect haemolymph (Goosey and Candy 1982) which clearly indicates that the target of OA must be located within the antennae. As previously noted, based on the anatomy of the antenna, we further concluded that the sensory apparatus located there is the most probable target site of OA and other substances released from neurohaemal release sites in the antennal heart. Since the antennae in *Schistocerca* are much shorter than in *Periplaneta*, the circulation time is certainly significantly shorter. Therefore, it seems conceivable that there are additional targets of the OA in the lateral region of the head capsule.

OA has been shown to be involved in the sensitivity modulation of the olfactory receptor neurons (ORNs) in the antennae of a number of insect species (*Periplaneta*: Zhukovskaya and Kapitsky 2006; Zhukovskaya 2007, 2008, 2012; Jung et al. 2013; several species of Lepidoptera: Pophof 2000, 2002; Grosmaître et al. 2001; Dolzer et al. 2001; Flecke and Stengl 2009; Hillier and Kavanagh 2015; Schendzielorz et al. 2015; *Apis*: Vergoz et al. 2009). All these studies refer to pheromone communication and a few papers consider non-pheromone odorants (Pophof 2002; Stelinski et al. 2003; Zhukovskaya 2012). OA was shown to increase the firing rate of pheromone-sensitive sensilla in various insect species (Pophof 2000; Grosmaître et al. 2001; Zhukovskaya and Kapitsky 2006; Zhukovskaya 2007; Flecke and Stengl 2009; Vergoz et al. 2009; Jung et al. 2013; Hillier and Kavanagh 2015). Different receptor cells of the same sensillum were shown to be modulated independently from each other, indicating that the OA receptors reside in the receptor neurons and not in the accessory cells (Zhukovskaya 2007). Evidence for OA receptors located in the antennal receptor cells has been presented for several species using in situ hybridization (von Nickisch-Rosenegk et al. 1996; Dacks et al. 2006; Brigaud et al. 2009; Jung et al. 2013; Lam et al. 2013). Experiments with injection of dsRNAs of OA receptors in *Periplaneta* provide additional support for the modulation of ORNs in the antennae (Jung et al. 2013). Furthermore, antennal ORNs have been shown to represent endogenous circadian oscillators (Merlin et al. 2007; review: Gadenne et al. 2016), and experiments indicate that these rhythms are modulated by OA in correlation with the insect's activity cycle (Flecke and Stengl 2009). Schendzielorz et al. (2015) demonstrated day-time-dependent changes in the concentration of OA in the antennae of *Manduca sexta*, which corresponds to the observation that changes in OA concentrations in the haemolymph of this moth are subject to a circadian rhythm (Lehman 1990). Furthermore, a tyramine-like immunoreactive fiber was

described in each fascicle of the antennal nerve in the flagellum region of *Manduca* (Schendzielorz et al. 2015). The authors speculated that these fibers might originate from neurons that mediate more rapid stimulus-dependent OA actions, thereby overruling the basic circadian rhythms of OA concentration changes in the haemolymph.

In conclusion, there is increasing evidence for a peripheral sensitivity modulation of the antennal ORNs by OA. For the task of long-term control via a hormonal pathway, the antennal heart represents the most suitable release site, since all haemolymph, which enters the antennae, must pass through this organ. Moreover, due to these anatomical constraints and conditions, a local regulation of the concentration of OA in the haemolymph within the antenna compartment is conceivable, which could be quite different from that of the general body cavity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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