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DIPLOMARBEIT

Titel der Diplomarbeit

Post-mating inhibition of sex pheromone responses in a male moth: search for a brain and/or sex gland factor

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie (A437)/Zoologie(A439)
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Wien, im Oktober 2010

Acknowledgements

I apologize to those who do not find themselves in the acknowledgments, the list would have been too long.

I am indebted to Christophe Gadenne and Sylvia Anton, who breached a trail to neurobiology for me. Merci beaucoup Christophe pour m'introduire dans le métier de physiologiste, qui n'est pas trop différent du métier de sorcier, et pour lire et re-lire (!) mes mémoires. Tu as beaucoup souffert.

Danke vielmals Sylvia, dass Du im Zuge eines Projektpraktikums von einem Land mit unbegrenzten Möglichkeiten berichtet hast. Andernfalls wäre ich wahrscheinlich aus typisch österreichischer provinzialistischer Borniertheit nie nach Frankreich gegangen.

Je remercie l'équipe des techniciens de l'unité PISC: Corinne, Fabien, Pascal et Cyril. Corinne je n'aurais jamais appris le français sans toi, merci beaucoup pour parler avec moi et m'expliquer où on trouve la levure en France. Merci beaucoup Fabien pour me dire les noms d'oiseaux. Merci beaucoup Pascal pour discuter avec moi de jazz classique et la façon correcte de hacher la canne. Je remercie particulièrement Cyril, car sans lui j'aurais oublié de vivre comme un être humain.

I thank all the people of the unit PISC to welcome me so friendly and open-minded.

Ich beneide Nina um ihr unglaubliches Geschick in Frankreich vernünftiges Bier aufzutreiben und danke für ihre Güte.

Je remercie Christophe H., Nicolas, Yamena et Elisabeth pour leur aide et leur connaissance de la bureaucratie française, une chose trop énigmatique pour un germanophone.

Je remercie Antoine, Alexandre, Christelle, David, Didier, Michel, Philippe et Virginie pour m'aider avec l'électrophysiologie.

Mein Dank gilt weiters den Menschen an der Universität Marburg, die mir den Aufenthalt dort möglich gemacht haben und besonders Max Diesner für sein analytisches Geschick im Umgang mit MALDI-Daten. Ich danke Martin, Basil, Martina, Jochen und Jörg für ihr wissenschaftliches Engagement und ihre Begeisterung für neurobiologische Belange.

Ich danke Harald Tichy, der die Aufgaben des Inneren Mentors an der Universität Wien übernommen hat.

Ich danke meiner Familie für die grenzenlose Unterstützung, und die grenzüberschreitenden Wege, die sie auf sich genommen haben.

Ich danke Claudia für die vielen Wege die sie auf sich genommen hat. Terima kasih Pogo. Aku cintamu, cewek tercantik di dunia.

This work was financially supported by INRA, and a scholarship (Stipendium für Kurzfristige Wissenschaftliche Aufenthalte) of the University of Vienna.

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List of Abbreviations

20-OH-E	20-Hydroxyecdysone
5HT	Serotonin
AL	Antennal Lobe
AMMC	Antennal mechanosensory and motor Centre
AN	Antennal nerve
AP	Action potential
ASG	Accessory sex gland
B	Artificial sex pheromone blend of <i>Agrotis ipsilon</i>
C	Controls for electrophysiology: mineral oil, hexane and blank filter paper
CA	Corpora allata
CC	Central complex
CF	Complete flight, behavioural response in the windtunnel
CNS	Central nervous system
DA	Dopamine
DopEcR	Dopamine-Ecdysone Receptor, membranous
EAG	Electroantennogram
EcR	Ecdysone Receptor, nuclear
ELISA	Enzyme linked immunosorbent assay
H	Heptanal
HB	Mixture of blend and heptanal
HPLC	high performance liquid chromatography
IN	Interneuron
JH	Juvenile hormone
L	Landing, behavioural response in the windtunnel
LN	Local neuron
M	Mated
M+2vBr	Mated male, injected with 2-brain-equivalents of virgin males
M+vASG	Mated male, injected with 1-ASG-equivalent of virgin males
MALDI-TOF	Mass spectrometric analysis, Matrix Assisted Laser Desorption/Ionisation - Time of Flight
MB	Mushroom Body, Corpora pedunculata
NM	No movement, behavioural response in the windtunnel
OA	Octopamine
OE	Oesophagus, oesophageal canal
OG	Ordinary glomerulus
OL	Optic Lobe
OBP	Odorant-binding protein
ORN	Olfactory receptor neuron
PBAN	Pheromone biosynthesis activating neuropeptide
PBP	Pheromone-binding protein
PC	Protocerebrum
PEI	Post-ejaculatory Interval
PF	Partial flight, behavioural response in the windtunnel
PI	Pars intercerebralis
PN	Projection neuron
PSP	Pheromonostatic peptide
RF	Random flight, behavioural response in the windtunnel
SOG	Suboesophageal Ganglion
SP	Sex peptide
SPR	Sex peptide receptor
TA	Tyramine
USP	Ecdysone Receptor, nuclear
V	Virgin
V+2mBr	Virgin male, injected with 2-brain-equivalent of mated males
V+2vBr	Virgin male, injected with 2-brain-equivalent of virgin males
V+mASG	Virgin male, injected with 1-ASG-equivalent of mated males
V+vASG	Virgin male, injected with 1-ASG-equivalent of virgin males
W	Walk, behavioural response in the windtunnel

Abstract

Olfaction is used by most animals to find adequate food sources and sexual partners. Newly-mated males of the moth *Agrotis ipsilon* experience a post-ejaculation interval (PEI), during which they are not attracted to the female-produced sex pheromone. This period lasts until the next night when the sensitivity is fully restored, and allows the males to refill their reproductive accessory sex glands for a potential new mating. This behavioural plasticity is accompanied by a reduced sensitivity of projection neurons (PN) in the primary olfactory centre, the antennal lobes (AL). This observed PEI could be due to a down- or up-regulation of neuroregulatory peptides, biogenic amines or ecdysteroid hormones.

Whereas there is no mechanism known to trigger PEI in insect males, a period of non-receptivity following mating was demonstrated in females of many insect species (e.g. Diptera, Lepidoptera). The female reproductive refractory phase is induced by proteins such as the sex peptide (SP) derived from the male accessory sex glands (ASGs), which are transferred during the copulation and act through the neuronal pathway via specific receptors. It might be possible that similar factors could have comparable effects in males.

In order to elucidate the origin of the observed PEI in *A. ipsilon* males, the effects of injections of brains or ASGs on the physiology of virgin and mated males were examined by windtunnel experiments.

Two main hypotheses were tested i) virgin males express in their tissues a substance that renders them sensitive to the female pheromone, which is no longer produced after mating; ii) mated males produce a substance as a result of mating that induces the PEI. Additionally, putatively differing peptide profiles of brains of virgin and mated males were compared by means of MALDI-TOF.

The results show that the behaviour of mated males could not be restored by the injection of tissues from virgin males. Contrarily, injections of ASGs, but not brains, of mated males significantly reduced the behavioural response of virgin males. Lastly, preliminary MALDI-TOF results show that the peptide profile is not drastically changed after mating.

Thus it is concluded that the male ASGs produce substances that not only affect the female's behaviour, but also act on the male itself. As males show changes in their behaviour similar to unreceptive females, it is presumed that a SP-like peptide induces the male PEI. Alternatively, the PEI could be triggered by dopamine (DA) or the insect hormone 20-Hydroxyecdysone (20-OH-E), as these substances have been shown to be of great importance in male sexuality in both vertebrates and insects.

1. Introduction

1.1. The insect brain

The ancestral state of the insect nervous system consists of a ventral nerve cord with paired ganglia in each segment. Advancing in insect evolution, the segmental structure of the paired ganglia is reduced due to the fusion of the segments. The first three ganglia are fused in all insects to form the brain. The three following ganglia are fused to form the suboesophageal ganglion (SOG).

The brain is commonly regarded as the main organ for collection and processing of sensory information, whereas the SOG is considered mainly as motor centre for the mouthparts and as centre for the transmission of information from the brain.

Three anatomically and functionally distinct areas can be distinguished in the insect brain, the protocerebrum, the deutocerebrum and the tritocerebrum.

1.1.1. *The protocerebrum*

The protocerebrum receives and processes sensory input from the eyes. Apart from neuropils required for the perception and integration of visual information, the protocerebrum features other substructures: the central body, the mushroom bodies and the lateral horns.

The central complex is a structure that receives information from the lateral and the median eyes, but is not a strictly visual area (Homberg 2008 and references therein). In *Drosophila melanogaster*, parts of the central complex are crucial for the maintenance of locomotion behaviour (Martin et al. 1999). Specific neurons in the central complex of *Schistocerca gregaria* respond to polarized light, which is used for orientation (Vitzthum et al. 2002).

The mushroom bodies or Corpora pedunculata (MBs) are of conspicuous shape and well studied (e.g. Dujardin 1850, Flögel 1876, Vowles 1955). Structure and organization of MBs are highly conserved within insect orders (Strausfeld et al. 1998). The MBs receive information from the deutocerebral antennal lobes (ALs) as well as other multimodal sensory inputs (Anton & Homberg 1999, Zars 2000). Hence MBs are crucial for the processing and the response to olfactory information (DeBelle & Kanzaki 1999 and references therein). However, they also play a role in locomotion control, by controlling the cessation of walking activity (Martin et al 1998). MBs are also involved in short-term memorization, e.g. of odours (Heisenberg 1998). In *Drosophila*, individuals with ablated mushroom bodies showed an impaired ability to learn (Heisenberg 2003). Pascual & Preat (2001) found MBs to be essential not only for the short-term memory, but also for the long-term memory.

The lateral horns are widely regarded as secondary olfactory processing centres, as they too receive input from AL projection neurons (Anton & Homberg 1999).

1.1.2. *The deutocerebrum*

The deutocerebrum comprises two neuropils, the ALs that receive olfactory input and the dorsal lobes, receiving mechanosensory projections (Anton & Homberg 1999). The dorsal lobes are also referred to as AMMC, antennal mechanosensory and motor centre, as they are also innervated by antennal motoneurons (Homberg et al. 1989). ALs are somewhat spheric and are usually well separated from the rest of the brain (Anton & Homberg 1999) (Figure 1).

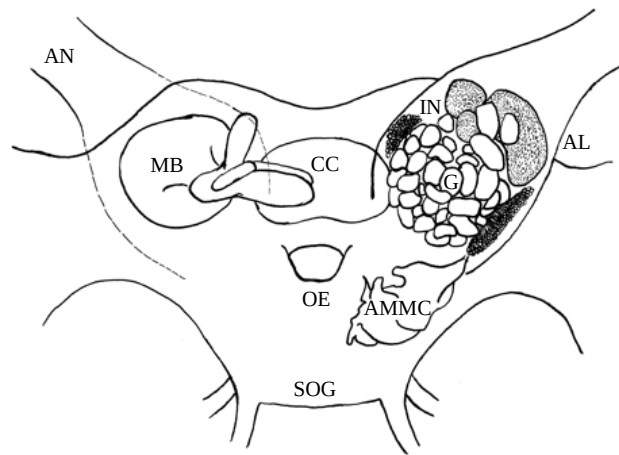


Figure 1. Frontal view of a moth brain without optic lobes. The central olfactory system comprises the antennal nerve (AN), leading olfactory information from the antennae to the antennal lobes (AL), and the antennal mechanosensory and motor centre (AMMC). Information is further sent to the protocerebral mushroom bodies (MB) and the central complex (CC), which are higher centres of integration and learning. Within the ALs, somata of interneurons (IN) are arranged around the glomeruli (G). In *Agrotis ipsilon*, three enlarged glomeruli in the ALs form the macroglomerular complex (MGC), shown as speckled glomeruli. Further abbreviations: OE, oesophagal canal; SOG, suboesophagal ganglion. Figure not to scale. After Anton & Homberg 1999, Lei et al. 2001 and Greiner et al. 2004.

1.1.3. *The tritocerebrum*

The tritocerebrum is connected to the mouthparts and the oesophagus, and has also connections to other brain areas as well as to the ventral cord (Rajashekhar & Singh 1994). Gustatory and mechanosensory information is being processed and spread from the tritocerebrum to the higher integration centres (Farris 2008).

1.2. Insect olfaction

Olfaction can be described as the detection and identification of airborne or waterborne chemical substances. In contrast to another variety of chemoreception, gustation or taste, it works at distance.

Chemoreception can be considered the most primordial sense, as bacteria and protometazoa respond to changes of their chemical environment (Macnab & Koshland 1972, Kung & Saimi 1982, Segall et al. 1986, Wadhams & Armitage 2004). Most animals use olfaction to find food sources, sexual partners, and to avoid peril. Odours can be used for long-distance, as well as for short-distance communication (Bossert & Wilson 1963).

For intraspecific communication animals produce and release pheromones. Pheromones can be used for social communication by eusocial insect societies, or for sexual communication to find a mating partner. In many moth species, females emit pheromones and males follow the odour plume upwind to eventually reach the female and mate (Shorey 1973, Kennedy & Marsh 1974, Cardé & Baker 1984, West-Eberhard 1984, Baker et al. 1988, Baker 1989, Vickers et al. 1991, Vickers 2006). In some species, females respond to the pheromone emitted by conspecifics, which triggers the concerted release of pheromone (e.g. Weissling & Knight 1996, Lim & Greenfield 2007, Lim et al. 2007) or synchronizes oviposition behaviour (Palanaswamy & Seabrook 1978).

Pheromones were originally defined in 1959 by Karlson & Butenandt as “substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction“. Since then a great variety of pheromones have been described in insects (e.g. Arn et al. 1992, Byers 2005). They are semiochemicals deriving from the fatty acid biosynthesis (e.g. Tillman et al. 1999, Jurenka 2004). Pheromone components are constituted of long carbon chains that allow variability in their conformation. Sex pheromones consist usually of a blend of more than one component, and the ratio of single components determines the species-specificity of the sex pheromone (Priesner 1986, Arn et al. 1986). In moths, this blend consists usually of 3 to 6 components (Wyatt 2003). For example, the migrant moth *Agrotis ipsilon* uses a female-emitted sex pheromone consisting of three main components: (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc) and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) (Picimbon et al. 1997, Gemenio & Haynes 1998).

However, many species share the same components, and males are often sensitive to pheromone components of other species. Therefore, many moth species use components that inhibit the response of other species to the sex pheromone (e.g. Vickers et al. 1991,

Mustaparta 1996, Gemenio et al. 2006). Pheromones of sympatric species are necessarily different, as sex pheromones serve to distinguish species, but may be equivalent in allopatric species (West-Eberhard 1984, Baker 2008). However, sex pheromones may differ in their composition within species (Olsson et al. 2006a, b; Kárpáti et al. 2008). Restrictions for the use of pheromones arise from the limited number of aliphatic molecules that can be synthesized by the females (reviewed by Byers 2005). All these attributes render the pheromonal communication highly specific.

1.2.1. *Detection*

To be able to respond adequately to the pheromone, males of many insect species have developed unique antennal structures that are distinct from the female antennae. For instance in the genus *Agrotis* males have developed bipectinate antennae, whereas females antennae are filiform (Carter 1984). Male antennae of species relying on pheromones are equipped with a high number of sensilla.

There are different kinds of sensilla that can be distinguished by their shape and function. The sensory functions of sensilla are highly diverse and depending on their design (Schneider 1964, Zacharuk 1980, Keil 1997, Galizia & Rössler 2010). Olfactory sensilla include sensilla trichodea, sensilla basiconica and sensilla coeloconica (Keil 1999). An olfactory sensillum is a porous cuticular protuberance housing the dendrites of up to three bipolar olfactory receptor cells surrounded by auxiliary cells that provide the chemical environment required for their function, the sensillar lymph (Zacharuk 1980, Keil 1999).

1.2.2. *Perception*

Odour molecules enter the sensilla via the pores and are then transported to the dendrites of the olfactory receptor neuron (ORN) by odorant-binding proteins (OBPs) (Stengl et al. 1992). For pheromones a specialized class of transport proteins, the pheromone-binding proteins (PBPs) are used (Vogt & Riddiford 1981). PBPs are highly abundant in the sensilla trichodea, that are thus pheromone sensitive (Laue et al. 1994, Kaissling 1996). Sensilla that are not specialized on the perception of pheromones contain general OBPs in high numbers (Steinbrecht et al. 1995). Once the odorant molecule is bound to the OBP, the complex is transported to the dendrite, where it generates a membrane potential by binding to a receptor. Thereafter, the odorant molecule is metabolized and deactivated by odorant degrading enzymes (Stengl et al. 1992). The mechanisms that lead to the membrane potential have been well described (e.g. Stengl et al. 1999). Briefly, the complex odorant molecule-OBP triggers

the depolarization of the ORN, either via second messenger cascade or via ligand-triggered ion channels (Stengl et al. 1999, Galizia & Rössler 2010).

In the antennae, axons from ORNs, mechanosensory neurons and further sensory neurons (e.g. hygroreceptive, thermoreceptive or contact-chemoreceptive) converge to the antennal nerve (AN), which leads to the ipsilateral AL (e.g. Homberg et al. 1989). Mechanosensory fibers from the antennae bypass the antennal lobe, whereas olfactory fibers spread throughout the glomeruli of the AL (Anton & Homberg 1999). The ALs are subdivided into anatomical and functional subunits, the so-called glomeruli (Anton and Homberg 1999). ORNs expressing the same kind of olfactory receptors project into the same glomeruli, hence revealing topographic odour representation (Hansson & Christensen 1999). Glomeruli house synaptic contacts between peripheral and central neurons. The input signal from the peripheral ORNs is integrated in the AL by interactions with local neurons (LNs) and projection neurons (PNs), and modulated by centrifugal neurons. PNs send the integrated information to the higher brain centres (output) (Anton & Homberg 1999). PNs receive input from ORNs and LNs equivalently, projections from PNs leave the AL via the inner, the middle or the outer antennocerebral tract and innervate parts of the mushroom bodies and the lateral protocerebrum (Hansson & Anton 2000). Centrifugal neurons have various input and output regions and may be modulatory in their function (Hansson & Anton 2000).

The glomeruli of the ALs are usually arranged around a core of interneuron neurites, and are flanked laterally and medially by the somata of interneurons (Anton & Homberg 1999). These subunits are separated from each other by glia, and are species-specific in their number and arrangement (Hansson & Anton 2000). Functionally, single glomeruli are targeted by specific groups of ORNs that all are sensitive to the same type of pheromonal or non-pheromonal odours (Christensen & Hildebrand 2002). Males develop specialized glomeruli in the macroglomerular complex (MGC) for the processing of pheromone information, which are homolog to unspecialized glomeruli in females (e.g. Koontz & Schneider 1987, Schneiderman & Hildebrand 1985, Rospars 1988, Anton & Homberg 1999, Rospars & Hildebrand 2000). In some noctuid moths such as *Agrotis segetum* and *A. ipsilon*, the male MGC is reported to consist of 4 distinct compartments (Hansson et al. 1994, Gemenio et al. 1998, Greiner et al. 2004). The other glomeruli, so-called ordinary glomeruli, respond to a variety of different odorants and encode the information perceived from the ORNs spatially and temporally (Hansson & Anton 2000). Research has recently focussed on the interaction of general odorants and pheromones (e.g. Ochieng et al. 2002, Reddy & Guerrero 2004, Yang et al 2004, Party et al. 2009).

The sensory information from the antennae and the ALs is transmitted to the protocerebrum and the higher centres of the brain by several antennocerebral tracts (Anton & Homberg 1999). Here, the information about the perceived odour molecules is further processed. The lateral horns and the MBs are of great importance for olfaction, as the lateral horns play a role in eliciting basic olfactory-guided behaviour and the MBs are required for associative olfactory learning (e.g. Heisenberg 2003, Masse et al. 2009).

In insects, many species respond to odours to find food (e.g. Myers & Walter 1970, Zhu et al. 1993, Syed & Guerin 2004) or adequate oviposition sites (e.g. Landolt & Molina 1996, Witzgall et al. 2005, Reisenman et al. 2009).

Orientation towards an odour source follows similar patterns in many arthropods (Vickers 2000). In general, they use a zig-zag pattern in their movements to stay in the plume and screen by moving approximately perpendicularly to the direction of the flow that carries the odour molecules (Baker 1989, Baker & Vickers 1994, Vickers 2000). In moths using pheromones, males use structures on the ground to estimate ground speed and can therefore modify their flight speed accordingly (Kennedy & Marsh 1974, Murlis et al. 1992). Challenges for the males that follow pheromone plumes are the anarchic plume structure as well as antennal adaptation to the pheromone (Baker et al. 1988, Murlis et al. 1992, Daly & Figueredo 2000).

1.3. Olfactory plasticity

Neuronal plasticity refers to the ability of any given nervous system to change in structure and function (Kolb & Wishaw 1998). In insects, olfactory plasticity has been shown to occur as a function of age/developmental stage, mating status, pre-exposure to odours or environmental conditions.

Plasticity in the perception and response to certain olfactory stimuli is crucial for insects, as not all stimuli are biologically relevant throughout the life history. For example, larvae may respond to different plant odours than adults, and sexually immature adults should not be attracted to courting mature adults. Additionally, different pheromones can be used for social communication throughout the development of an insect. In *S. gregaria*, the larvae produce different aggregation pheromones than the adults (Obeng-Ofori 1993, Obeng-Ofori et al. 1994, Torto et al. 1994). In *A. ipsilon*, production of pheromone by females is age-dependent (Gemeno & Haynes 2000, Xiang et al. 2010). Also, the mating status can modulate the response of insects to odours. Mated females should be more sensitive to plant odours than virgin ones to find adequate sites for oviposition. Newly-mated males that have emptied their

accessory sex glands (ASGs) during copulation should delay the next behavioural response to pheromone in order to increase their chances of a successful re-mating. The sensitivity to sex pheromone can further be modulated by pre-exposure to biologically relevant odours (e.g. MafraNeto & Baker 1996, Devaud et al. 2001, Anderson et al. 2003, Stelinski et al. 2004, Anderson et al. 2007). Lastly, changes in the environmental conditions may lead to a difference in perception and, thus, response to odours (cf. Baker & Cardé 1979, Linn & Roelofs 1992, Linn et al. 1996, Merlin et al. 2007).

However, only age- and mating dependent olfactory plasticity shall be discussed here.

1.3.1. Age-dependent plasticity

Age-dependent plasticity of olfaction, characterized by modulation of the behavioural response to odours, has been shown to take place in different parts of the olfactory system. Studies have shown a peripheral olfactory plasticity in some species (e.g. Domingue et al. 2006, Lemmen & Evenden 2009). However, the age-dependent olfactory plasticity has been shown to occur mainly at the brain level in various species. This age-dependent plasticity is linked with size increase in ALs of ants (Gronenberg et al. 1996), flies (Devaud et al. 2003), and honeybees (Wang et al. 2005), and with AL neuron sensitivity in moths (Anton & Gadenne 1999).

Age-dependent plasticity occurs also in other parts of the insect brain. In honeybees, MB volume increases with age, although this increase is mainly dependent on the task of an individual in the colony (Farris et al. 2001, Fahrbach et al. 2003, Maleszka et al. 2009). Dufour & Gadenne (2006) found a population of proliferating cells in the MBs of *A. ipsilon*, thus showing adult neurogenesis, which could be involved in olfactory plasticity.

Males of a variety of insect species reach their sexual maturity a few days after eclosion. This coincides with the maximal sensitivity to the female-produced pheromone (Adeesan et al. 1969, Gadenne & Anton 2000, Greiner et al. 2002, Jarriault et al. 2009a). In migrant moths such as *Pseudaletia unipunctata* and *A. ipsilon*, males are not sexually mature at the time of eclosion and become receptive to the female pheromone after a few days (Turgeon et al. 1983, Gadenne et al. 1993).

In *Spodoptera littoralis*, older females exhibit higher sensitivity to plant odours than freshly emerged ones (Martel et al. 2009). However, in male *A. ipsilon*, the AL neuron response to “neutral” plant odours is not influenced by age (Greiner et al. 2002).

Mechanisms of age-dependent plasticity

Various neuromodulators such as hormones and biogenic amines have been shown to be involved in the mentioned effects of age-dependent plasticity. Juvenile Hormones (JH), produced by the corpora allata (CA), a retrocerebral gland, regulate larval molting and metamorphosis (e.g. Williams 1959a, b), but also many phases of adult reproduction (Wyatt & Davey 1996, Riddiford 2007). In particular, JH regulates the decreased response to sex pheromone of diapausing males in the moth *Catoptilia fraxinella* (Lemmen & Evenden 2009). This hormone was also shown to control the pheromone production of *P. unipunctata* and *A. ipsilon* (Cusson & McNeil 1989, Gadenne et al. 1993, Picimbon et al. 1995).

In males of the moth *A. ipsilon*, JH controls sexual maturation and thus behavioural and AL neuron response to the female pheromone (Gadenne et al. 1993, Duportets et al. 1996, Duportets et al. 1998, Anton & Gadenne 1999), but not the response to plant odours (Greiner et al. 2002). Males that do not produce JH due to ablation of the CA do not respond to pheromone (Gadenne et al. 1993).

The steroid Ecdysone and its active form, 20-Hydroxyecdysone (20-OH-E) are insect hormones that regulate metamorphosis and, putatively, act as sex hormones on the genitalia of males and females and the production of gametes (e.g. de Loof & Huybrechts 1998, de Loof 2006, Spindler et al. 2009). These hormones also influence the adult nervous system. Ecdysone triggers neuroblast proliferation and the growth of neurites in the MB of the cricket *Acheta domesticus* (Cayre et al. 2000). Growth and adult neurogenesis are affected by 20-OH-E (Prugh et al. 1992, Gu et al. 1999). Fluctuations of 20-OH-E levels cause the expression of various genes in the brain of *Apis mellifera*, *D. melanogaster* and *Anopheles gambiae* (cf. Velarde et al 2009, and references therein). Concerning sexual behaviour, reports on *Drosophila* sp. suggest that both ecdysone and its receptors are essential for mediation and learning of successful courtship patterns in males (Ganter et al. 2007, Dalton et al. 2009, Ishimoto et al. 2009). In male *A. ipsilon*, preliminary experiments show that 20-OH-E might be involved in the maturation of the olfactory system. Injections of 20-OH-E into young immature males increased their behavioural response to sex pheromone compared to Ringer-injected males of the same age (Vitecek, unpublished data).

Biogenic Amines have also been shown to modulate the sexual behaviour in many insect species. Octopamine (OA) was discovered first in the salivary glands of *Octopus vulgaris*, a name-giving event for one of the major neuroeffectors in insects (Erspamer & Boretti 1951). OA, like tyramine (TA), is synthesized from tyrosine (Lange 2009). Both OA and TA are only found in a low number of neurons in the CNS of insects (Roeder 2005, and references

therein). However, OA and TA have highly significant effects in their life history, comparable to adrenaline/noradrenaline in mammals (Roeder 2005 and references therein). TA acts as paracrine substance on the Malpighian tubes of insects, and has antagonistic (i.e., inhibitory) effects compared to OA on muscle contraction, cAMP and Ca^{2+} levels, and locomotor activity (Osborne 1996, Roeder 2005, Lange 2009 and references therein).

OA has been found to have different effects on the physiology depending on the pathway: OA can act as a neuromodulator, as neurotransmitter and as neurohormone (Farooqui 2007 and references therein). In honeybees, OA is important for division of labour, olfactory memory, nutritional preference in foragers and leads to higher sensitivity to sensory inputs (e.g. Hammer & Menzel 1998, Schulz & Robinson 1999, Wagener-Hulme et al. 1999, Barron et al. 2002, Scheiner et al. 2006, Giray et al. 2007). Crickets treated with OA antagonists showed an impaired recall ability of appetitive memory (Mizunami et al. 2009). In the cockroach *Periplaneta americana*, topical application of OA on the antennae led to a decrease in the amplitude of EAG response to sex pheromone (Zhukovskaya 2007). In lepidoptera, OA has been reported to act on the behavioural response to sex pheromone (Linn et al. 1992, Linn et al. 1994, Linn et al. 1996), to induce flight activity (Vierk et al. 2009) and to increase the firing rate of ORNs in response to sex pheromone (Pophof 2000, Grosmaître et al. 2001). In *A. ipsilon* males, the presence of OA is important to optimize the behavioural response to sex pheromone (Jarriault et al. 2009a). Moreover, it enhances the AL neurons sensitivity in immature males, whereas mianserin, an OA antagonist, decreased both the behavioural and AL neuron sensitivity in sexually mature males (Jarriault et al. 2009a).

Serotonin (5-Hydroxytyramine, 5HT) is synthesized in vertebrates as well as in invertebrates from tryptophan (Blenau & Baumann 2001). It is found in specific parts of the insect brain and especially in ALs (e.g. Settembrini & Villar 2004, Hoyer et al. 2005, Sinakevitch 2008; for a review, see Schachtner et al. 2005). The effects of 5HT on the insect physiology are numerous. In *A. mellifera*, foragers exhibit higher brain 5HT levels than nursing bees (Schulz & Robinson 1999). Yet, brain 5HT levels are also influenced by the age of an individual bee (Wagener-Hulme et al. 1999). 5HT has been found to inhibit adaptation to a changing light-dark rhythm in *Drosophila* sp. (Yuan et al. 2005). The tobacco hornworm *Manduca sexta* shows variable levels of 5HT, with a maximum in ALs coinciding with the period of highest activity in these moths (Kloppenborg et al. 1999). Interestingly, this monoamine has been found to increase AL sensitivity and responsiveness to sex pheromone in this moth (Dacks et al. 2008, Kloppenborg & Mercer 2008). Similar results have been reported on *Bombyx mori*, as moderate doses of 5HT enhance male sensitivity to bombykol

(Hill et al. 2003, Gatellier et al. 2004). However, injections of 5HT in the antenna decreased the spontaneous spike frequency in the noctuid *Mamestra brassicae* (Grosmaître et al. 2001).

1.3.2. Mating dependent plasticity

Females of many species exhibit remarkable changes subsequent to mating. Behaviour, protein synthesis and pheromone synthesis of honeybee queens change drastically after a successful mating (Kocher et al. 2008, 2009). In many species, newly-mated females show a change in receptivity, evidently caused by transfer of seminal substances or mechanical stimulation (e.g. Eberhard 1996, Chapman et al. 1998, Miyatake et al. 1999, Wedell 2005, Klowden 2006, Kocher et al. 2009). These changes originate from products of the male accessory sex glands (ASGs) (Gillot 2003). For example, in drosophilid flies, males transfer so-called sex peptides which lead to post-mated behavioural and gene expression changes in females (Chapman et al. 2003, Kubli 2003, Chapman & Davies 2004, McGraw et al. 2004, Carvalho et al. 2006, Kubli 2008, Isaac et al. 2010). In *Aedes aegypti*, ASG proteins have been reported to affect the host-seeking behaviour of mated females (Fernandez & Klowden 1995). In *Ceratitis capitata*, the mediterranean fruitfly, virgin females prefer the male-produced pheromone, whereas mated females of the same species prefer host-fruit odours (Jang 1995, 2002). In female *S. littoralis*, EAG responses to a variety of odours change significantly after mating (Martel et al. 2009). In *Lobesia botrana* only mated females orient towards their host plant, *Vitis vitifera* (Masante-Roca et al. 2007).

In the heliothine moth *Helicoverpa zea*, male sex gland factors induce the change from “virgin” to “mated” behaviour in females (Kingan et al. 1993). Sex pheromone production strongly decreases after mating, due to a male-produced peptide (Kingan et al. 1995). *Helicoverpa armigera* females show the same status after injection of *D. melanogaster* sex peptide, suggesting the presence of a similar peptide in noctuid moths (Fan et al. 1999).

Mating in insects is preceded by distinct and complex courtship patterns and involves the transfer of a spermatophore in some groups (Scudder 1971). This spermatophore is produced by the ASGs, which are emptied during mating (e.g. Duportets et al. 1998, Gadenne et al. 2001, Jimenez-Perez & Wang 2004). To remate successfully, males need to replenish their glands for the formation of a new spermatophore (He & Tsubaki 1992, Wolfner et al. 1997, Gadenne et al. 2001, Jimenez-Perez & Wang 2004). Virgin males have also a higher reproductive output than mated males (Torres-Vila & Jennions 2005). The ASGs are of significant importance for male reproductive success (cf. Leopold 1976, Marshall 1982, Chen 1984, Boggs 1990, Park et al. 1998, Gwynne 2008).

Mated males of the noctuid species *A. ipsilon* cease to respond to the female sex pheromone (Gadenne et al. 2001). This coincides with the drastic reduction of AL PN sensitivity after mating (Gadenne et al. 2001). However, antennae remain sensitive to the pheromone, as EAG responses and single sensillum recordings of antennal ORNs do not differ between virgin and mated males (Gadenne et al. 2001, Barrozo et al. 2010a). This decrease in sensitivity after mating seems to be restricted to pheromone processing as responses to plant odours are not affected in mated males (Barrozo et al. 2010a). Hence *A. ipsilon* males seemingly experience a refractory period with a decreased sexual sensitivity.

A similar refractory period has been reported in *Protohermes grandis* (Megaloptera), *Gryllus bimaculatus* (Orthoptera), a variety of tettigoniid species, *Spalangia endius* (Hymenoptera), *Plutella xylostella* (Lepidoptera), and *Ostrinia nubilalis* (Lepidoptera), in which mated males avoid females after mating (Simmons 1990, Hayashi 1993, Royer & McNeil 1993, Matsumoto & Sakai 2000, Reddy & Guerrero 2000, Fischer & King 2008).

This is most likely due to the high costs of mating in insects. In females, mating reduces the fitness due to a variety of factors (e.g. Reynolds & Gross 1990, Chapman et al. 1993, Siva-Johty et al. 1998, Watson et al. 1998, Johnstone & Keller 2000, Stutt & Siva-Johty 2001, Rolff & Siva-Johty 2002, Orsetti & Rotuwski 2003, Morrow & Arnqvist 2003, Martin et al. 2004, Wigby & Chapman 2005). However, females may benefit from copulation, especially when high paternal investment occurs (Vahed 1998, Arnqvist & Nilsson 2000, Bonduriansky 2001, Fedorka & Mousseau 2002, Edvardsson 2007, Gwynne 2008, McNamara et al. 2008).

Mating, and especially paternal investment are highly costly for males. Males suffer costs from sperm competition (cf. Simmons et al. 1993, Snook 1998, Lamunyon 2000, Martin & Hosken 2002, Wedell et al. 2002, Snook 2005, Solensky & Oberhauser 2009) and production of ejaculate substances (cf. Leopold 1976, Thornhill 1976, Marshall 1982 and references therein, Rutowski et al. 1983, Bissoondath & Wiklund 1996). In males providing large nuptial gifts, mating success is positively correlated with the size of nuptial gifts (Butlin et al. 1987, Gwynne 1988, Gwynne 1991, Vahed 1998, Lehmann & Lehmann 2000, Gwynne 2008, McNamara et al. 2009). Incorporation of male ASG substances that are transferred during copulation and that increase female fitness occurs in many groups, including lepidoptera (Boggs 1981, Bownes & Partridge 1987, Boggs 1990 and references therein, Markow et al. 1990, Pitnick et al. 1991, Hayashi & Kamimura 2002). Fertility (i.e. amount of sperm transferred) and spermatophore size (i.e. amount of ASG-produced substances) of multimated males is decreased in comparison to monogamous males of many lepidopteran species (Kaitala & Wiklund 1994, Kaitala & Wiklund 1995, Torres-Vila & Jennions 2005 and

references therein, Marcotte et al. 2007). Hence, male costs of mating may be of great significance for the incidence of a sexual refractory phase in insects.

Mechanisms of mating-induced plasticity

In mammals, males show a distinct period of reduced sexual activity and sensitivity after ejaculation (e.g. Yilmaz & Aksu 2000). This period, usually referred to as the post-ejaculatory interval (PEI), is mainly regulated by 5HT and dopamine (DA), but involves other hormones and biogenic amines (Hull et al. 1986, Mas et al. 1995, Bancroft 1999, Hull et al. 2004, Bancroft 2005). However, knowledge is scarce about the mechanisms involved in the regulation of the PEI in insect males.

In *A. ipsilon*, the JH biosynthesis activity does not change after mating, suggesting that this important hormone might not be involved in the regulation of the PEI (Duportets et al. 1998). However, moths of many species store high amounts of JH in the ASGs and transfer JH during copulation to the females, presumably to enhance egg production (Cusson et al. 1999, Pszczolkowski et al. 2006). The biosynthesis of proteins in ASG of male *Drosophila* sp. can be enhanced after mating by topical application of JH (Wolfner et al. 1997). A similar activity could be hypothesised in moths, as male ASGs are depleted of their proteins directly after mating in *A. ipsilon* (Duportets et al. 1996, Duportets et al. 1998). Additionally, experiments of OA and 5HT injections into mated *A. ipsilon* males showed that these two biogenic amines could not restore the behavioural response to sex pheromone of mated males, despite increasing AL neuron sensitivity (Barrozo et al. 2010b).

In insect males, ecdysteroids are produced in the testis and are essential for the formation of genitalia and to induce spermatogenesis (de Loof & Huybrechts 1998, Simonet et al. 2004). Moreover, males of *Anopheles gambiae* produce and transfer 20-OH-E to the female during mating (Pondeville et al. 2008). In *Drosophila* sp., 20-OH-E was found to enhance ASG biosynthesis in mated males, similar to JH (Herndon et al. 1997). However, 20-OH-E has been reported to interrupt release of sperm into the seminal vesicle in male *S. littoralis* (Polanska et al. 2009 and references therein). Therefore, the production of ecdysteroids in the ASG could also act on the male itself in order to induce the PEI. An increase in the level of 20-OH-E might be involved in the regulation of the PEI of many insect males.

1.4. The model insect: *Agrotis ipsilon*

Agrotis ipsilon (Hufnagel) is a migrant noctuid moth (Lepidoptera: Noctuidae) and a cosmopolitan pest. It inhabits as seasonal migrant all parts of the world except Iceland, Greenland and the Antarctic.

Imagines of *A. ipsilon* have a wingspan from 35 to 45 mm. The forewings have a brownish base colour, that differ between individuals from light brown to dark brown. Three forewing stigmata (reniform, orbicular and claviform) are outlined in black. A short Y-shaped strak extends from the reniform stigma towards the termen. The hindwings are ashen-white, with brown veins and terminal line. Males and females can be distinguished by inspection of antenna, as males possess bipectinate antennae whereas females bear simple filiform antennae (Fig. 2) (Carter 1984).

Larval characters and diagnostic features are described in Carter, 1984.

Larvae of *A. ipsilon* typically behave as cutworms; they are polyphagous and feed on a great variety of plants. Foodplants include many genera from Poaceae, Chenopodiaceae, Asteraceae, Brassicaceae and Solanaceae (http://www.agroatlas.ru/en/content/pests/Agrotis_ipsilon (23/04/2010, 13h30)). Before pupation they undergo 6 to 7 larval stages and pupate in the ground in 3 to 12 cm depth (Showers 1997).

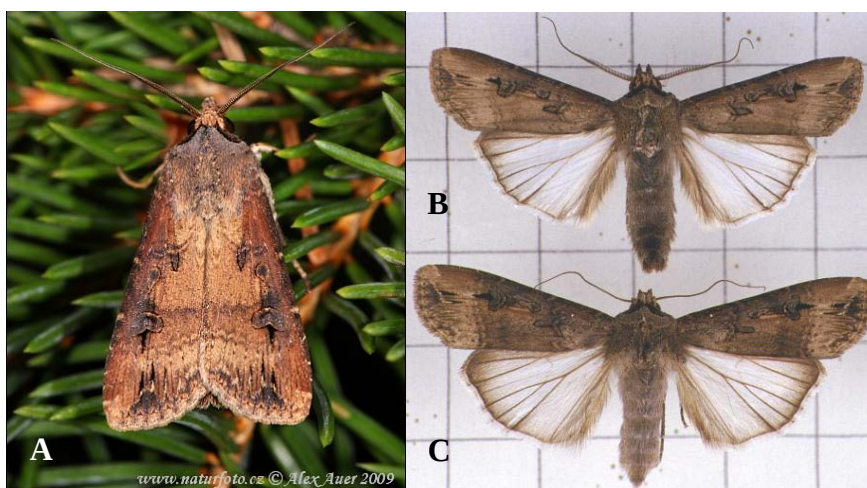


Fig. 2: *Agrotis ipsilon*. A, B: male, C: female. Note the males' bipectinate antennae. Photographs: A: <http://www.naturephoto-cz.com/agrotis-ipsilon-picture-12501.html> (11/04/2010, 11h10); B & C: http://www.funet.fi/pub/sci/bio/life/insecta/lepidoptera/ditrysia/noctuoidea/noctuidae/noctuinae/agrotis_ipsilon.jpg. (11/04/2010, 11h10).

A. ipsilon revealed to be a good insect model to study the plasticity of olfaction. Main research has focussed on maturation of olfactory processing and the influence of biogenic amines on the response to sex pheromone (cf. Anton et al. 2007, Jarriault et al. 2009a, 2009b,

Duportets et al 2010, Jarriault et al. 2010). However, the PEI is well described in this species. It is a period that lasts until the end of the nocturnal activity phase, in which the males do not respond to the sex pheromone (Gadenne et al. 2001, Vitecek unpublished data). Anton et al. (2007) review the changes in the post-mated *A. ipsilon* male as follows: ASGs are empty after mating, sensitivity of AL PNs is reduced and there is a reduced response to sex pheromone. Antennal sensitivity and JH biosynthesis are not affected by mating, and the males do not recover from the mating-induced changes until the following night (Anton et al. 2007). Moreover, biogenic amines do not seem to be involved in the post-mating transient inhibition of sex pheromone response (Barrozo et al. 2010b).

Interestingly the sex pheromone becomes inhibitory after mating (Barrozo et al. 2010a). Both virgin and mated males respond to plant odours, but mated males do not orient towards plant odours if sex pheromone is added to the plant odours.

Other studies have focussed on the mechanisms of the PEI. An increased level of 20-OH-E in post-mated males of *A. ipsilon* has been reported (Siaussat unpublished data). On the central level, an increased expression of the dopamine-ecdysone receptor DopEcR has been shown (Duportets, unpublished data). However from the hitherto obtained data, nothing is yet known on the identity of the factor(s) inducing this PEI in mated males of *A. ipsilon*.

1.5. Aims of the present study

Male *A. ipsilon* cease to behaviourally respond to the female sex pheromone after mating. This is, as mentioned earlier, due to changes in the sensitivity of PNs in the ALs (Gadenne et al. 2001, Barrozo et al. 2010a, b). Whereas the influence of mating on females is well known in insects, there is no mechanism known to trigger PEI in males.

The lack of response to the sex pheromone following mating could result from the action of one or many specific proteins/peptides in the brain. These proteins could be produced in the brain and directly or indirectly induce the observed lack of receptivity. However, it is also possible that this factor originates from the ASGs, as these glands are of importance for the reproductive success (cf. Chapter 1.3.2).

The present study was performed to assess whether behavioural changes can be elicited in virgin and mated males by injections of extracts of brains or ASGs. The effects of the injection of tissues were examined by wind-tunnel experiments. Virgin males received tissues from mated donors in order to possibly decrease their response to the sex pheromone. Mated males received injections of tissues from virgin donors in order to possibly restore the lost responsiveness to the sex pheromone.

By injecting tissues of virgin males into mated males we addressed the hypothesis that mated males lack a factor present in virgin males that is required to respond to pheromone. Indeed it was previously shown that when either JH or OA are missing, the behavioural response is impaired (cf. Gadenne et al. 1993, Jarriault et al. 2009a).

Virgin males received injections of tissues from mated males to focus on the second hypothesis: newly-mated males produce a factor, absent in virgin males, which elicits the PEI. For example, in females of many species the sex peptide, received from the male during mating, can trigger the post-mating change in receptivity (cf. Fan et al. 1999).

Additionally, the occurrence incidence of peptides was compared by means of MALDI-TOF. Putatively, peptide contents in tissues of virgin and mated males could differ in quantity and/or in quality. This seems likely, as in post-mated *A. ipsilon* the observed reduced sensitivity of AL PNs could be due to a down- or up-regulation of neuroregulatory peptides.

2. Material and Methods

2.1. Insects

Adult *A. ipsilon* were used in the experiments. The insect colony originates from field catches in southern France and wild insects are introduced each Spring. The animals were reared on an artificial diet (Poitout & Buès 1974, Appendix) in individual cups until pupation. Pupae were sexed and males and females were kept separately in an inversed light/dark cycle (16 h light: 8 h dark photoperiod) at 22°C. Newly emerged adults were removed from the hatching containers every day and were given access to a 20% sucrose solution *ad libitum*. Day of emergence was considered as day-0. In all windtunnel experiments sexually mature 5-day old males were used.

2.2. Mating experiments

Pairing experiments were performed as described previously (Gadenne et al. 2001, Barrozo et al. 2010a, b). Briefly, virgin 5-day-old sexually mature males and virgin 3-day-old sexually mature females were individually paired in cylindrical plastic containers before the onset of scotophase in a mating room under a 16L:8D photoperiod and at $22 \pm 1^\circ\text{C}$. Visual observation of matings was done every half hour during the mating period at mid-scotophase (i.e. from 14:00-17:00). Once males had mated (copulation lasts between one and two hours), they were removed from the pairing box and used for tissue collection or windtunnel experiments within one hour after the end of the copulation. Only males that transferred a spermatophore were used for further experiments. To be sure that the male introduced a spermatophore during mating, all mated females were checked for the presence of a spermatophore.

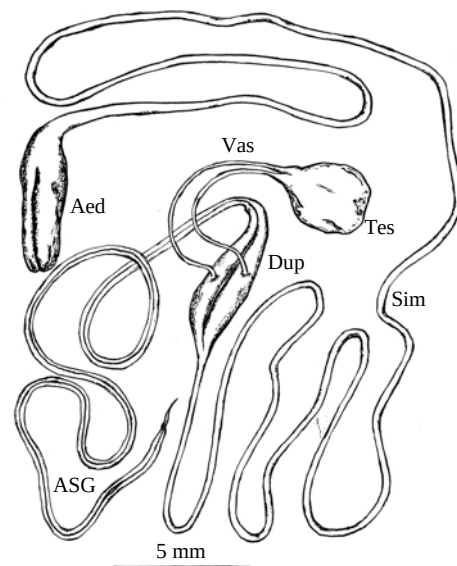
2.3. Stimuli

The artificial pheromone blend contained (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc), and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc) (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio of 4:1:4 (Picimbon et al. 1997, Gemenio & Haynes 1998). For all behavioural tests, 10 ng of pheromone blend were used. Prior experiments showed that this dose gave the best behavioural results with sexually mature virgin males (Barrozo et al. 2010b).

2.4. Tissue collections

Brains and ASGs (Fig. 3) were dissected from mated and virgin males in the second half of the scotophase, when this species shows the highest reproductive activity (Gadenne et al. 1993, Xiang et al. 2010). Tissues of newly-mated males were dissected out within one hour following the end of mating. Dissections were conducted in Ringer's solution (see Appendix). ASGs were dissected out prior to brains to ascertain the mating status of the insects. In virgin males, ASGs are thick and strongly reddish coloured, on the contrary ASGs of newly-mated males are thin and translucent. Removed tissues were then immediately placed in Eppendorf tubes in liquid nitrogen and stored at -80°C .

Figure 3. Male genitalia of *Agrotis ipsilon*. Aed, Aedagus; ASG, Accessory Sex Glands; Dup, Ductus ejaculatorius duplex; Sim, Ductus ejaculatorius simplex; Tes, fused testes; Vas, Vas deferens. The ASGs are paired glandular tubules connected with the Ductus ejaculatorius duplex. Modified after Gemeno et al. 1998.



2.5. Tissue preparation and injection

The collected tissues were homogenized with a Polytron several times in Ringer's solution on ice. To facilitate injections, $1\mu\text{l}$ of Ringer's solution was added for each brain, and $2\mu\text{l}$ for each ASG. Ringer's Solution was added to the frozen tissues in Eppendorf vials, tissues were then allowed to thaw on ice. Thawed tissues were crushed five times on ice for 1s each until all solid contents were crushed (ASGs of virgin males occasionally required further repetitions). ASGs from virgin males were further centrifuged at 4°C at 1500 rpm in a Hettich EBA-12 centrifuge, and the supernatant was collected for injections.

Crude homogenates as well as supernatants were stored on ice prior to injection. Roughly, 20 brains homogenized in $20\mu\text{l}$ Ringer's solution yielded about $20\mu\text{l}$ crude solution, of which usually $18\mu\text{l}$ could be used, the rest could not be removed from the vial. ASGs of virgin males that were homogenized and centrifuged produced in general the same amount of supernatant as was expected from the added amount of Ringer's solution (i.e. 10 ASGs homogenized with

20µl Ringer's produced 20µl supernatant). ASGs of mated males were completely homogenized and yielded about twice the amount of crude solution as was expected from the amount of Ringer's added (e. g. 10 ASGs homogenized in 20µl of Ringer's produced roughly 40 µl of crude solution). This yielded higher volumes of ASG crude solution from mated males than from virgin males. Therefore higher amounts of ASG crude solution from mated males were injected in order to obtain one ASG-equivalent.

For the injections of brain extracts, each male received a dose of 2 brain-equivalents (equal to 2µl of crude solution). ASG extracts were given in a dose of 1 ASG-equivalent (equal to 2µl of supernatant/ 4µl of crude solution). All injections were administered into the abdomen.

2.6. Treatments

Both virgin and mated males were injected with brain and ASGs extracts. Virgin males were injected with tissues from mated males, and with tissues from virgin males as control injections. Mated males were injected with tissues of virgin males. Untreated (non injected) virgin and mated males served as controls (Table 1).

Table 1. Treatment groups and tissue injections in *A. ipsilon* males. Virgin animals received injections of tissues of mated and virgin animals, mated males were injected with tissues of virgin animals. Mated males and virgin males were used as controls as well as virgin males injected with tissues of virgin males.

Test Animal	Donor	Tissue	Abbreviation
Virgin	Mated	Brain	V+2mBr
Virgin	Mated	ASG	V+mASG
Mated	Virgin	Brain	M+2vBr
Mated	Virgin	ASG	M+vASG
Virgin	Virgin	Brain	V+2vBr
Virgin	Virgin	ASG	V+vASG
Mated	-	-	M
Virgin	-	-	V

2.7. Windtunnel experiments

The windtunnel experiments were run in a room under the same reversed light photoperiod, temperature and humidity conditions as in the room holding the pupae and adults (see above) (Gadenne et al. 1993). The tunnel room was illuminated using three red-light lamps, producing a maximum of 22 lux inside the tunnel. The windspeed was measured as

0,34 ms⁻¹. The horizontal flow of the odour plume was assessed optically by use of incense sticks. After this, the tunnel was set active for 12 h to eliminate disturbing odours.

All test insects were placed in cylindrical test cages, which were installed in the room at least 3 h prior to the behavioural test. In the semicylindrical 150 cm-long windtunnel, a cage with a test insect was placed on a platform at a height so that the odour plume could enter the cage. A filter paper containing the synthetic pheromone blend was installed upwind at the other end of the tunnel. The filter papers were changed after three consecutive tests. Cages and filter paper-carrying devices were cleaned daily after use to avoid odour saturation on the material.

Each male was tested only once for 180 s and was thereafter discarded. The behavioural response of the males to the sex pheromone was quantified in two categories with three subcategories each:

Non responding:

No movement (NM): The male did not leave the cage.

Walk (W): The male left the cage, walked on the floor, sometimes reaching the source holder and climbing onto it.

Random flight (RF): The male left the cage, and was observed flying inside the tunnel and showing no response to the odour plume.

Responding:

Partial Flight (PF): the male took off and performed a specific zig-zag flight horizontally in the odour plume towards the odour source for about 75% of the distance between the odour source and the cage.

Complete Flight (CF): Same as above but reaching the proximity of the source without landing.

Landing (L): The male flew all the way from the cage to the odour source on which it landed.

All animals that did not fly were tested for their flight ability. Only males capable of flying were included in the data set.

2.8. MALDI-TOF mass spectrometry

This technique was used to perform a comparative peptide analysis of brains from virgin and mated males. Brains were dissected out in buffer (0.01M Phosphate buffer; 0.15M NH_4Cl), and their functional subunits (ALs, CAs, optic lobes (OLs), pars intercerbralis (PI) cells, protocerebrum (PC), and SOG) were sampled. Tissues were transferred by means of glass capillaries onto a clean MALDI-TOF plate and spread out to obtain a thin layer of tissue. In a second step, the tissues were covered with a matrix of cinnamic acid (ca. 0.5 g dissolved in 2 ml 30% EtOH (100%), 30% MeOH (100%), 40% TFA (0.1%)). The prepared plate was kept in a vacuum desiccator with silica gel, to avoid humidification and thereby decomposition of the tissues.

Peptide contents of tissues were sampled in a 4800Plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Darmstadt, Germany) in positive ion reflector mode. Peptides were ionized by a laser beam, and their mass was estimated automatically by their ionization time of flight. Spectra represent the resolved monoisotopic masses in the mass range m/z 500-3500.

The obtained spectra were analysed manually using DataExplorerTM. Peptides were identified by a) their conspicuous mass divergence, b) comparing several spectra obtained from sampling the same parts of the brain of several animals and selecting peptide-like molecules, which were found in more than 70% of all samples. The mass of the presumed peptides was compared with data from literature (Audsley & Weaver 2003, Audsley & Weaver 2005, Utz et al. 2007, Neupert et al. 2009) to find matching peptides.

2.9. Statistical methods

Statistical analysis of behavioural responses of males to the sex pheromone was performed by comparing the relative proportion of responding males (partial flight + complete flight + landing) between groups, using Chi-Square tests ($p \leq 0.05$). Because the data on peptidomics are only preliminary, statistical methods were not applied to these results.

3. Results

To test the possibility that an internal factor could switch off the behavioural response after mating, virgin and newly-mated males were injected with tissues originating from newly-mated and virgin males, respectively, and tested for their behavioural response to sex pheromone.

Overall, 515 males were tested. In the non-injected control groups, 133 virgin males (V) and 60 mated males (M) were tested. The sample of virgin males was subsampled to 65 individuals in order to improve statistical stability. Of mated males injected with virgin brains (M+2vBr) or ASGs (M+vASG), 65 and 49 animals were tested, respectively. To test the effects of control injections into virgin males, virgin males injected with brains (V+2vBr) and ASGs (V+vASG) of virgin males, 51 and 53 animals were tested, respectively. To assess the effects of injections of tissues from mated males into virgin males, 50 virgin males injected with brain extracts of mated males (V+2mBr), and 54 virgin males injected with ASG extracts of mated males (V+mASG) were tested in the windtunnel (Table 2).

3.1. Sex pheromone-mediated behaviour of virgin and newly-mated males

Sexually mature virgin 5-day old males were tested during 3 min in the windtunnel at mid-scotophase (between 14:00h and 17:00h) for their response to a dose of 10 ng synthetic sex pheromone blend. From the 133 virgin males tested, 65 were subsampled by standardized randomization. Nearly all males (96.9%) left the cage and were observed to fly in the windtunnel. Forty-five (69%) of the males showed an oriented flight towards the pheromone source by performing a typical zig-zag flight. Of these, 9 males landed on the odour source (Tab. 2).

Mature males paired with females were observed for occurring copulations. Once couples had finished copulation, the males were removed from the pairing box and submitted to windtunnel experiments within one hour after the end of copulation. Of the 60 newly-mated males tested, only 2 (3%) responded to the pheromone. The remaining 58 males did not move (33), or performed random flights (25) (Tab. 2). The response of newly-mated males was highly significantly different from that of virgin controls ($\chi^2=57.75$, $df=1$, $p<0.001$) (Fig. 4).

3.2. Behavioural response of newly-mated males injected with tissue extracts of virgin males

Newly-mated males were injected in mid-scotophase with tissue extracts from virgin males (Brains or ASGs) within 1 h after the end of copulation, and tested 1 h later in the windtunnel. Sixty-five newly-mated males received a dose of 2 virgin brain-equivalents (M+2vBr), and 49 newly-mated males were injected with a dose of 1 virgin ASG-equivalent (M+vASG).

Brain extracts and ASG extracts could not restore the inhibited behavioural response to sex pheromone in newly mated males. The response of control newly-mated males (3%), newly-mated males injected with brain-extracts (1.5%) and newly-mated males injected with ASG-extracts (0%) does not differ ($\chi^2=0.43$ for the comparison of control mated males and brain-injected mated males, $\chi^2=1.66$ for control mated males and ASG-injected mated males, and $\chi^2=0.79$ for brain-injected mated males and ASG-injected males; $df=1$ and $p>0.05$ for all groups). Mated males injected with 2 virgin brain-equivalents responded significantly less to the pheromone than control virgin males ($\chi^2=65.13$, $df=1$, $p<0.001$), as did mated males injected with 1 virgin ASG-equivalent ($\chi^2=56.05$, $df=1$, $p<0.001$). The response of mated males injected with virgin tissues did not differ between test groups receiving virgin brains or ASGs ($\chi^2=0.79$, $df=1$, $p>0.05$) (Tab. 2, Fig. 4).

3.3. Behavioural response of virgin males injected with tissue extracts of mated males

Virgin males were injected in mid-scotophase with tissue extracts of mated males and were tested in the windtunnel one hour after the injection. Fifty virgin males received injections of 2 mated brain-equivalents (V+2mBr), and 54 males were injected with 1 mated ASG-equivalent (V+mASG). Injections of brain extracts did not change the males' response to the sex pheromone blend significantly, as compared to control virgin males (78.2% to 69.2%, $\chi^2=1.1$, $df=1$, $p>0.05$).

Injections of ASG extracts reduced the number of responding males significantly, as compared to the response of control virgins (29.6% to 69.2%, $\chi^2=18.51$, $df=1$, $p<0.001$). Significantly more virgin males injected with 2 mated brain-equivalents responded to the pheromone compared to virgin males receiving injections of 1 mated ASG-equivalent ($\chi^2=24.38$, $df=1$; $p<0.001$). However, more virgin males receiving mated brain or ASG-extracts responded to the sex pheromone than mated control males ($\chi^2=65.03$, $df=1$, $p<0.001$ and $\chi^2=14.78$, $df=1$, $p<0.001$, respectively).

Tissues of virgin males were injected into virgin males to serve as control-injections. The response of virgin males injected with either virgin brain extracts (V+2vBr, 84.3%) or virgin

ASG extracts (V+vASG, 56.6 %) did not differ from that of virgin controls (69.2%) ($\chi^2=2.62$ for the comparison of control virgin males and brain-injected virgin males, $\chi^2=2.01$ for control virgin males and ASG-injected virgin males; $df=1$ and $p>0.05$ for both groups) (Fig 3).

Interestingly, the response of virgin males injected with virgin ASG extracts was significantly lower than that of virgin males that received virgin brain extracts ($\chi^2=8.09$, $df=1$, $p<0.05$). Also the response of virgin males that received injections of virgin brain extracts was higher but not statistically different from that of control virgin males. Virgin males injected with mated or virgin brain extracts showed no difference in their response to the pheromone ($\chi^2=0.30$, $df=1$, $p>0.05$) (Fig. 4).

3.4. Further comparison of groups

Response patterns of mated males and virgin males that were injected with virgin tissues differ highly significantly. M+2vVr and M+vASG males responded less than V+2vBr and V+vASG respectively ($\chi^2=71.94$, $df=1$, $p<0.001$ and $\chi^2=39.34$, $df=1$, $p<0.001$; respectively).

The observed number of responding V+2mBr males is significantly higher than the number of V+vASG males responding to pheromone ($\chi^2=5.33$, $df=1$, $p<0.05$). Significantly fewer M+2vBr and M+vASG males responded than V+2mBr males ($\chi^2=72.84$, $df=1$, $p<0.001$, and $\chi^2=63.06$, $df=1$, $p<0.001$; respectively) and than V+2vBr males ($\chi^2=80.01$, $df=1$, $p<0.001$ and $\chi^2=69.57$; $df=1$, $p<0.001$; respectively).

V+2vBr and V+vASG males responded significantly more than V+mASG males ($\chi^2=29.49$, $df=1$, $p<0.001$, and $\chi^2=7.94$, $df=1$, $p<0.001$).

The response pattern of V+mASG males was significantly higher than that of M+2vBr and M+vASG males ($\chi^2=19.01$, $df=1$, $p<0.001$, and $\chi^2=17.19$, $df=1$, $p<0.001$; respectively).

V+vASG males responded significantly more often to the pheromone than M+2vBr and M+vASG males ($\chi^2=45.7$, $df=1$, $p<0.001$, and $\chi^2=39.29$, $df=1$, $p<0.001$; respectively).

Table 2. Data obtained from windtunnel experiments. Abbreviations: % flight, Percentage of flying animals; % orient., percentage of responding animals; for further abbreviations, see Chap. 2.6.. Overall, 447 animals were used for statistical analysis. Percentage of responding males is high for virgin males, except for virgin males injected with mated ASGs. Mated males do not respond to the pheromone. Flight activity of mated males is lower than that of virgin males.

Test group	Non Responding			Responding			N	% orient.	% flight
	NM	W	RF	PF	CF	L			
V	2	0	18	23	13	9	65	69,23	96,92
M	33	0	25	2	0	0	60	3,33	45
V+2mBr	3	0	8	21	12	6	50	78	94
V+mASG	14	1	23	2	6	8	54	29,62	72,22
V+2vBr	0	2	7	26	8	8	51	82,35	96,07
V+vASG	11	1	11	18	6	6	53	56,60	82
M+2vBr	56	4	4	1	0	0	65	1,53	7,69
M+vASG	45	1	3	0	0	0	49	0	6,12

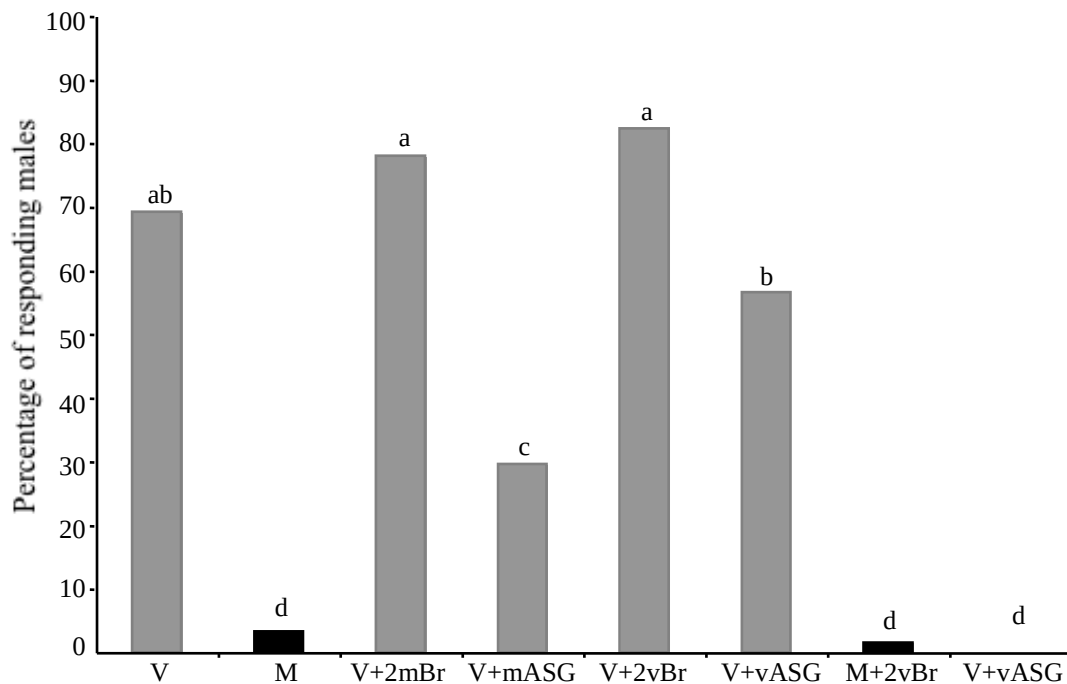


Figure 4. Effects of brain/ASGs injections on the behavioural response of *A. ipsilon* male moths to sex pheromone. For abbreviations, see Chapter 2.2.3. and 2.2.4.. Sample size: V, n=65; M, n=60; V+2mBr, n=50; V+vASG, n=54; V+2vBr, n=51; V+vASG, n=53; M+2vBr, n=65; M+vASG, n=49. Grey bars: virgin males, black bars: mated males. Bars with the same letters are not statistically different.

3.5. Peptidomics

In a period of 10 days, 90 samples were processed. 7 samples were obtained by using one female of unknown age. Of the rest, 38 samples were obtained from 2 virgin and 2 mated day-6 males. Forty-five samples were taken from day-5 males, 3 of which mated prior to dissection (Tab. 3).

Table 3. Processed samples. AL, single antennal lobe; CAs, corpora allata; OL, single optic lobe; PC, one half of a protocerebrum; PI-cells, neurosecretory cells situated medially on the pars intercerebralis; SOG, Suboesophageal ganglion.

Insects (n)	AL	CAs	OL	PC	PI-cells	SOG
Female (1)	2	-	2	2	-	1
Day-5 Virgin Male (4)	8	3	-	6	3	3
Day-5 Mated male (3)	6	3	-	6	3	3
Day-6 Virgin male (2)	4	1	4	4	-	1
Day-6 Mated male (2)	4	1	4	4	-	2
Total	24	8	8	22	6	10

3.5.1. *Peptides and Spectra*

In the ALs, I could identify 4 peptides. These are Cydiastatin 3 (peptide mass 925.5 Dalton), Cydiastatin 4 (909.5 Da), Helicostatin 9 (1393.7 Da), and Allatotropin (1487 Da).

Further peptides that were identified by coworkers on the project are presented and discussed in the appendix (App. A).

4. Discussion

Virgin males are highly sensitive to the sex pheromone, in contrast to mated males. Mated males are scarcely attracted to a source of pheromone. This perfectly confirms previous results showing an inhibition of behavioural response to sex pheromone in newly-mated *A. ipsilon* males (Gadenne et al. 2001, Barrozo et al. 2010a, b).

The behavioural response of mated males can not be restored by the injection of virgin tissues. These results indicate that it is most likely not the absence of a specific factor that causes the lack of behavioural response to sex pheromone in newly-mated males.

However, the behavioural response of virgin males can be significantly reduced by the injection of ASGs from mated males. These results show that there is most likely a factor that is produced or enhanced in the ASGs during and after the copulation that triggers the post-copulatory refractory phase in newly-mated males. Therefore the hypothesized presence of a substance inducing the typical behaviour (PEI) in mated males can be confirmed.

Brains of mated males and tissues of virgin males (both brains and ASGs) have no effect on the physiology of virgin males.

4.1. Strong inhibition of sex pheromone responses in post-mated males

In many animals, mating elicits changes in the physiology of individuals. In the case of the male *A. ipsilon*, the animals cease to respond to the female-produced pheromone. Results of Barrozo et al. (2010a) suggest furthermore an inhibitory effect of sex pheromone on mated males.

Our study confirms previous results showing a strong inhibition of behavioural response to sex pheromone in newly-mated *A. ipsilon* males (Gadenne et al. 2001, Barrozo et al. 2010a, b). From all the males tested in the wind tunnel after copulation, only two individuals were observed to show an oriented response towards the odour source. Interestingly, these two males were starved, i.e. not given access to a sugar solution in the pairing compartment. Moreover, these starved mated males showed a higher flight activity than mated males that had access to sugar solution (Vitecek, unpublished data). Lastly, the two responding mated males showed a straight upstream flight into the plume, in contrast to virgin males which showed a typical zig-zag flight (see also Baker & Vickers 1997).

Newly-mated males not only do not respond to the sex pheromone, they also show a lower flight activity than virgin males and are less active. Barrozo et al. (2010b) recently showed that the sex pheromone, which is attractive for virgin males, becomes inhibitory to newly-mated males: mated males are inhibited to respond to the sex pheromone, and therefore do not

fly. The higher flight activity of starved mated males suggest that they were in need for food. Starved animals could therefore use the pheromone traces (indicating the presence of conspecific females) to detect food. Females are indeed most likely to attract males in vicinity to adequate larval food plants (for a review, see Shorey 1973). Most of these plants, like linden, also provide food for adult insects.

For this study it was hypothesized that the strong olfactory behavioural switch-off observed after mating could originate from a factor present in the brain or the ASGs, which could be either down-regulated or up-regulated after mating. It is suggested that this factor could be a peptide or a neuromodulator acting on the sensitivity of the AL neurons (cf. Gadenne et al. 2001).

Injections of tissues extracted from virgin males did not affect the receiving mated males. One-brain-equivalent and two-ASG-equivalent extracts from virgin males injected into newly-mated males did not restore the behavioural response of the injected males. Similarly, injections of neuromodulators such as biogenic amines (octopamine and serotonin) into newly-mated males could not restore the behavioural response to sex pheromone (Barrozo et al. 2010a).

Altogether, these results suggest that the mating-induced olfactory inhibition in newly mated males does not originate from the decrease of a factor present in tissues of virgin males. Therefore, the presence of a substance originating from the ASGs in mated males is proposed, which affects the observed inhibition to the sex pheromone.

To test the possibility that mating could induce the production of an inhibitory factor, brains or ASGs of mated males were injected into virgin males in order to reduce the expected behavioural response. Injections of a two-brain-equivalent extract of mated males did not influence the behavioural response of virgin males. On the contrary, injections with the ASGs of mated males (one-ASG-equivalent) significantly reduced the response of virgin males.

However, there was no complete absence of behavioural response in the injected virgin males, indicating that the injections with ASG-extracts could not induce the complete PEI, which is observed after copulation. Nevertheless the results indicate that the tissues that induce the PEI are most probably the ASGs. The effect of other tissues (testes, and other parts of the male reproductive system) has not yet been examined.

The potential to elicit the behavioural change is also present in the ASGs of virgin males, as controls (virgin males injected with ASGs of virgin males) showed a reduced response. Therefore it is concluded that during copulation and within a period of at least one hour after

the copulation, the ASGs emit or produce in a higher rate one or many substances that induce the PEI in *A. ipsilon* males.

4.2. Mating-induced up-regulation of an inhibitory factor originating from the ASGs

4.2.1. Peptides

The mating-induced changes in behavioural responses to sex pheromone in *A. ipsilon* males resemble those induced by the sex peptide in females after copulation. Females that have copulated show a reduced receptivity towards other males, tend to avoid further mating attempts by males, and start oviposition (Gemeno et al. 2007, Yapici et al. 2008, Clyne & Miesenböck 2009, Fricke et al. 2009).

In the yellow fever mosquito *A. aegypti* a peptide present in the ASGs of the males, and transferred into the female during copulation, has been shown to inhibit the female host-seeking behaviour after mating (Fernandez & Klowden 1995, Lee & Klowden 1999). In the female mediterranean fruit fly *Ceratitis capitata* there is a preferential switch as a result of mating. Mated females stop to be attracted to the male-emitted sex-pheromone and start to be attracted to fruit odours (Jang 1995).

In *D. melanogaster*, injections of HPLC fractions of male ASGs into females led to the isolation of the 36-amino acid sex peptide (SP or Acp70A) (Chen et al. 1988, Chapman et al. 2003, Liu & Kubli 2003). This peptide was shown to elicit the post-mating change in female *D. melanogaster*. Injection of physiological amounts of purified or synthetic SP cause virgin females to become unreceptive to mating attempts by males and stimulate egg production for a period of 1-2 days.

In noctuid moths, mating elicits a post-copulatory state in females that lasts until the next night and which is characterized by the cessation of pheromone production and release (Raina 1988, Raina 1989). This transient inhibitory period is regulated by a factor, which is present in the female haemolymph after mating (Raina 1989). The factor was identified and sequenced as the pheromonostatic peptide (PSP), deriving from the male ASGs (Kingan et al. 1995). Further was shown that PSP inhibits the release of the pheromone biosynthesis activating neurohormone (PBAN) in mated females, thus inhibiting production and release of pheromone (Rafaeli 2009, and references therein). Sex peptide as well as other seminal peptides seem to be very similar within insects, as post-mating behaviour can be induced in *H. armigera* females by injection of both *D. melanogaster* and *H. armigera* SP and seminal peptides (Fan et al. 1999, Fan et al. 2000, Nagalakshmi et al. 2007).

In male insects, ASGs of *H. armigera* and *D. melanogaster* have been shown to be depleted of their seminal proteins such as SP after mating (Nagalakshmi et al. 2007, Sirot et al. 2009). Moreover, mating and hormonal triggers (20-OH-E or JH) induce ASG gene expression in male *D. melanogaster* (Herndon et al. 1997).

Sex peptide or other seminal peptides have no reported effect on the male organism. However, it is likely that, in addition to emptied ASGs in mated *A. ipsilon* males (cf. Duportets et al. 1998), also SP is depleted, as in mated *H. armigera* males. It is therefore highly unlikely that this substance could play a role in the post-copulatory refractory phase observed in newly mated *A. ipsilon* males.

Nevertheless, ASG peptide profiles of virgin and mated *A. ipsilon* males should be assessed using approaches like HPLC (cf. Nagalakshmi et al. 2004, Nagalakshmi et al. 2007), ELISA (cf. Sirot et al. 2009), or MALDI-TOF (cf. Schachtner et al. 2010).

4.2.2. Hormones

In preliminary experiments, neurotransmitters such as serotonin and octopamine could be eliminated as possible effectors of the PEI in *A. ipsilon* (cf. Barrozo et al. 2010a). However, JH and 20-OH-E remain to be tested as possible effectors of the PEI.

In a variety of lepidopteran species, JH is stored in the male ASGs and transferred to the female during copulation (cf. Shirk et al. 1980, Park et al. 1998, Cusson et al. 1999). In male *A. ipsilon*, JH biosynthetic activity is not influenced by mating (Duportets et al. 1998). However, as JH is reported to enhance sensitivity to sex pheromone in this species (Gadenne et al. 1993, Jarriault et al. 2009a) and the contents of the ASGs are depleted during mating (Duportets et al. 1998), it seems unlikely that JH is the inhibiting factor that induces the PEI.

Ecdysteroids have been detected in the reproductive tissues of many insect species (de Loof & Huybrechts 1998, Simonet et al. 2004, Pondeville et al. 2008 and references therein). In the mosquito *A. gambiae*, males produce and transfer 20-OH-E to the female during mating (Pondeville et al. 2008). In *S. littoralis* 20-OH-E has been found to interrupt the release of sperm into the seminal vesicle (Polanska et al. 2009 and references therein). If 20-OH-E had a similar effect in *A. ipsilon*, one would expect a strong increase of the ecdysteroid level shortly after mating. Preliminary results show that haemolymph of newly-mated males contains higher amounts of 20-OH-E compared virgin males (Siassaut unpublished data). However, this is contradictory to preliminary experiments, which show that the sensitivity of submature

A. ipsilon males is enhanced by injection of 20-OH-E, (Vitecek unpublished data). Further research will focus on 20-OH-E levels in ASGs of virgin and mated males.

4.3. Proposed mode of action of the ASG inhibitory factor

The proximate function of the PEI is clear: a transient inhibition of sex pheromone response allows newly-mated males that have depleted their seminal proteins to stop their reproductive behaviour in order to conserve energy and materials and to replenish the ASG proteins for further successful mating.

As AL neuron sensitivity to sex pheromone is strongly decreased after mating (Gadenne et al. 2001), the ALs are most likely the major target organ for the ASG inhibitory factor(s). Response latency, action potential intensity, and durations of excitatory and inhibitory phases are indeed reduced (Barrozo et al. 2010a, b).

All these changes can be affected by neuroregulatory peptides. Although SP can be excluded as a potential ASG inhibitory factor (see above), there is still the possibility that this or a similar peptide binds to a specific receptor in the brain, thus inducing a cascade event leading to the PEI. In *D. melanogaster* females, a SP receptor (SPR) has been cloned and acts to induce the post-mating switch in the reproductive behaviour (Yapici et al. 2008). This receptor is expressed in the female reproductive tract and in the CNS, mainly in the suboesophageal ganglion. In males of *D. melanogaster*, intriguingly, the SPR is absent in the reproductive tract, but present in the CNS, in a distribution similar to that of SPR in the female CNS (Yapici et al. 2008). Moreover, the SPR is highly conserved across insects (Yapici et al. 2008). A receptor similar to SPR could therefore exist in male *A. ipsilon* and could elicit the PEI in this species.

The haemolymphatic level of 20-OH-E is higher in mated males of *A. ipsilon* (see Chap. 1.5, 4.2). In *A. ipsilon*, a variety of candidates for 20-OH-E receptors have been detected and cloned. There is a membrane receptor, DopEcR, binding 20-OH-E and dopamine (DA), and two nuclear receptors for 20-OH-E (EcR, USP) (Duportets et al. unpublished data). DopEcR has been located exclusively in the CNS of *A. ipsilon* males, and its expression level is higher in mated males compared to virgin males (Duportets et al. unpublished data).

An increased level of DopEcR could increase the neuronal sensitivity to DA, which could lead to the observed PEI (cf. Stahl, 1999). Whether DA alone or the combined effects of DA and Ecdysone on the DopEcRs induce the changes, remains hitherto unknown. However, as 20-E injections increased the responsiveness to the sex pheromone of sexually mature virgin males, it is hence most unlikely that ecdysteroids should have an inhibiting effect on the

nervous system. Further, inhibition of Ecdysone receptors via the injection of Cucurbitacine also reduces the level of response of mature males (Gadenne unpublished data). This is why DA is proposed as the main effector of the switch to the PEI that occurs during/after mating. The proposed effect of DA in the insect nervous system differs from reported effects of DA in mammal sexuality (cf. Pfaus et al. 1990, Bancroft 2005, Krüger et al. 2005).

4.4. Analysis of peptides in brains of virgin and mated *A. ipsilon* males

The preliminary results, using MALDI-TOF analysis of tissues of virgin and mated males, do not show striking differences between the groups. However, the sample size is hitherto too small to interpret the data. Work is now in progress to sample more tissues and thus prevail a peptide fingerprint of *A. ipsilon*. In a second step, analysis and comparison of mated male tissues with the peptide fingerprint will hopefully reveal presence or absence of certain peptides, or fluctuations in the proportions of certain peptides that are due to mating.

4.5. Challenges

The major aim of this study was to elicit the typical post-mating behaviour by injection of a variety of tissues. It soon showed that to obtain results, the animals had to be convinced to participate and cooperate. However, this is a rather complicated matter. The animals seemingly do not mate willingly, if they are going to be dissected. Also mated animals for windtunnel experiments proved to be of equal stubbornness. From the animal's point of view, it is more than logic to exclude oneself from the experiments, as they are tiring and dangerous.

Little was the effect of poetry on the benevolence of the animals, nevertheless this might be due to the fact that all poetry recited was in German or English. As the population was founded close to Bordeaux it is more than likely that these animals can not be reached by these languages, and that, for the sake of the study, I should have learned more french.

The major challenge was to obtain enough insects to either test or dissect. As the caveman was constantly held in terror by primordial predators, I was constantly in awe of low individual numbers.

In a period in which the institute was suffering low numbers of insects, I was given the chance to perform some electrophysiology on pheromone-specific antennal sensilla. This measure did eventually result in higher numbers, so that I could proceed with the windtunnel work (although the relationship of electrophysiology and individual numbers is not necessarily a causal one).

All other challenges encountered were of minor quality and could be solved within a few days time, e.g. a broken fan.

Dissection of tissues was not a hindering factor, as the methods applied are easy and fast, allowing the dissection of a maximum of 104 animals per day. However, I shall counsel every student not to do so, as this work at this rate is capable of eradicating every feeling of pity or sympathy.

If the numbers of insects apply, quite some can be tested daily in the windtunnel. It is possible to test a maximum of roughly 60 animals per day, but very tiring and rather exhausting. A day that yields 25 tested animals can be considered a good day from the point of a windtunnel physiologist.

5. Appendix

5.1 Artificial diet for *A. ipsilon* larvae

Boil 420 ml of water, add whilst stirring 24 g of Agar-Agar. Let the mixture boil for about 10 min. During that time, prepare a solution of 2.1 g benzoic acid and 2.1 g nipagine (Para-Hydroxy Benzoic acid ester) in about 10 ml ethanol. Prepare separately a mixture of 168 g maize flour, 45 g yeast, 42 g wheat germ and 6 g ascorbic acid.

To the boiling water, add 600 ml of cold water. Then add, whilst stirring, the solution of Benzoic acid and Nipagine. Slowly add, whilst stirring with a mixer the prepared mixture of flour and the other dry ingredients.

Pour the diet into rearing cups and let them rest until the mixture has hardened.

5.2. Ringer's Solution (so-called Tucson Ringer)

Dilute in 1 l of distilled water: 8.76 g NaCl, 0.333 g CaCl₂, 0.224 g KCl, 2.29 g TES (buffer). Add NaOH to regulate pH (6.9). Add 8.55 g sucrose and store refrigerated.

5.3. Appendix A

A1. Preliminary results of MALDI-TOF mass spectrometry

A1.1. Mass Spectrometry of Antennal Lobes

Due to mating, the sensitivity of antennal lobe PN is reduced drastically (Gadenne et al. 2001). This ultimately leads to the PEI, during which newly-mated males do not respond to the female-produced sex pheromone (Gadenne et al. 2001, Barrozo et al. 2010a, Barrozo et al. 2010b). As the observed behavioural changes can be explained by the action of neuroregulatory peptides, it was presumed that changes in peptide composition could be observed in the ALs (Table A1).

There are several peptides, among those several previously described (Nässel 2002), that were not recorded in either virgin or mated males. In ALs of virgin males, Mas- γ SGNP and the sodium adduct of AKH were not recorded. Analysis of ALs of mated males did not record FLRF Amide III and Cydiastatin 2.

Table A1. Peptide list for ALs as obtained by MALDI-TOF mass spectrometry. Abbreviations: AKH*, sodium adduct of AKH; Mass (Da), peptide mass in Dalton; Rel. Ab., number of samples in which a given peptide was found. Number of samples processed: Virgin male ALs n=10, mated male ALs n=10. In ALs of virgin males, Mas- γ SGNP and the sodium adduct of AKH were not found. Mated male ALs lack FLRF Amide III and Cydiastatin 2.

Peptide Class	Peptide	Virgin		Mated	
		Mass (Da)	Rel. Ab.	Mass (Da)	Rel. Ab.
<i>FLRF-amide</i>	FLRF Amide III	880.50	10%	-	-
	FLRF Amide I	1229.60	50%	1229,63	70%
<i>Helicokinine</i>	Helicokinin II	847.50	70%	847,45	80%
<i>Helicostatine</i>	Helicostatin 5	911.50	100%	911,47	100%
	Helicostatin 1	934.40	50%	934,46	10%
	Helicostatin 9	1393.70	100%	1393,66	100%
<i>Cydiastatine</i>	Cydiastatin 4	909.50	100%	909,49	100%
	Cydiastatin 3	925.50	100%	925,48	100%
	Cydiastatin 2	2168.50	10%	-	-
<i>CAPA-like peptides</i>	CAP2b	975.50	20%	975,53	60%
	Mas-PVK-1/CAPA-1	975.54	20%	975,53	60%
	Mas-PVK-1/CAPA-1	992.57	50%	992,56	100%
<i>PBAN-like peptides</i>	Mas- γ SGNP	-	-	816,45	10%
	Agi- γ SGNP	964.50	20%	964,50	30%
<i>Allatotropine</i>	MANSE-AT	1486.70	100%	1486,71	100%
<i>Myoinhibitory peptides</i>	MIP VI	997.50	30%	997,50	50%
<i>CCAP</i>	CCAP	956.40	10%	956,44	10%
<i>Corazonin</i>	Corazonin	1369.70	10%	1369,66	10%
<i>Allatostatine</i>	Q1-MANSE-AS	1905.90	60%	1905,88	20%
<i>AKH</i>	AKH*	-	-	1030,49	10%

However, even though there are peptides that are differentially distributed, it is possible that this is due to methodological bias. A peptide that was found in one of ten samples might have been introduced by errors during the dissection. Indeed, as the relative abundance represents the numbers of samples containing a certain peptide, a great bias can be introduced by single samples, as the overall sample size is not very high. To conclude, there is probably no or little significant changes in the peptide composition in the ALs to be observed after mating.

A1.2. Mass spectrometry of the other brain tissues

Mass spectrometry of other tissues yielded sample sizes lower than ten and are therefore not discussed. However, peptides were also detected in the CNS (without ALs) of *A. ipsilon* males (Table A2).

Table A2. Preliminary peptide list of the male *A. ipsilon* CNS. Abbreviations as in Table A1, additional abbreviations: AKH**, potassium adduct of AKH; CA, Corpora allata; PC, Protocerebrum. Presence of peptides is indicated by a cross, absence by a minus sign.

Peptide Class	Peptide	Mass (Da)	CA		PC	
			Virgin	Mated	Virgin	Mated
<i>FLRF-amide</i>	FLRF Amide III	880.50	x	x	x	-
	FLRF Amide I	1230.00	x	x	x	x
<i>Helicokinin</i>	Helicokinin II	847.50	x	-	x	x
<i>Helicostatine</i>	Helicostatin 5	911.50	x	x	x	x
	Helicostatin 9	1394.00	x	x	x	x
<i>Cydiastatine</i>	Cydiastatin 4	909.50	x	x	x	x
	Cydiastatin 3	925.50	x	x	x	x
<i>CAPA-like peptides</i>	P20-28	936.50	-	-	-	x
	CAP2b	975.50	x	x	x	x
	Mas-PVK-1/CAPA-1	975.50	x	x	x	x
	Mas-PVK-1/CAPA-1	992.60	-	x	x	x
<i>PBAN-like peptides</i>	Agi- γ SGNP	964.50	x	x	x	x
<i>Allatotropine</i>	MANSE-AT	1487.00	x	x	x	x
<i>Myoinhibitory peptides</i>	MIP VI	997.50	x	x	x	x
	MIP III	1386.00	x	x	x	-
<i>CCAP</i>	CCAP	956.40	-	x	x	-
<i>Corazonin</i>	Corazonin	1370.00	x	x	x	x
<i>AKH</i>	AKH*	1031.40	x	-	-	-
	AKH**	1046.40	x	x	-	-
<i>Allatostatine</i>	MANSE-AS	1889.00	-	x	x	x
	Q1-MANSE-AS	1906.00	x	-	x	x

5.4. Appendix B: Plant odours block sex-pheromone detection in a male moth

B1. Introduction

In insects, olfaction is used to find food sources, and to localize sexual partners. Male moths are known for their ability to detect females emitting sex pheromone over long distances. The male search for female sex pheromones occurs in a background environment, which is rich, if not saturated, in plant odours. Therefore males meet the challenge of extracting the crucial pheromonal cue from a continuously changing odorant environment. Plant odours have been reported to strongly enhance the behavioural response of male *S. exigua* to sex pheromone (Deng et al. 2004). Also in the male codling moth *Cydia pomonella*, the behavioural response to the sex pheromone is higher when plant volatiles are added to the stimulus (Yang et al. 2004). Similar effects of plant volatiles on the behavioural response to sex pheromone have been found in the male grape berry moth *Eupoecilia ambiguella* (Büsser-Schmidt et al. 2009). This suggests synergistic effects of plant volatiles and sex pheromone in these species.

In the ALs, pheromonal and non-pheromonal (i.e. plant odours) information are separately processed. Pheromone-specific olfactory receptor neurons (ORNs) project into the MGC, whereas general ORNs project into the ordinary glomeruli (OG). However, also on this level, a synergy of plant odours and sex pheromone can be observed. In the silkworm, *B. mori*, a synergistic effect of plant odours and sex pheromone has been described on the response of AL interneurons (Namiki et al. 2008).

At the peripheral level, a synergistic effect of plant volatiles and sex pheromone has been found to occur in sex pheromone-specific ORNs in *H. zea* (Ochieng et al. 2002). On the contrary, antennal ORNs of *S. littoralis* have been reported to respond less to the sex pheromone if the sex pheromone is presented in mixture with plant odour (Party et al. 2009).

As a synergy on behavioural and central nervous level has also been described in *A. ipsilon* males (Barrozo et al. 2010a), we tested the effects of sex pheromone-plant volatile mixture on the responses of ORNs.

B2. Material and Methods

B2.1. Insects

Mature, 5-day old virgin *Agrotis ipsilon* males were used for single sensillum recordings. Larvae were reared on semi-artificial diet until pupation. Pupae were sexed. Males and females were kept separately in a reversed 8:16 L:D cycle at 22°C and 50% relative humidity.

B2.2. Preparation

All experiments were conducted in the scotophase as previously described (Jarriault et al. 2009). Shortly, males were chosen randomly for the tests and fixed in blocks of insulation foam. Tip recordings were performed in concordance with Kaissling & Thorson (1980). The antenna of interest was fixed using strips of adhesive tape. The sensilla of the antenna were cut at the very tip using sharpened forceps. A glass capillary containing a physiological solution (containing 172 mM KCl, 37,5 mM glucose, 10 mM Hepes, 3 mM MgCl₂, 1 mM CaCl₂, 25 mM NaCl; pH=6,5) was put over the cut tip of one sensilla. To avoid cristallization at the tip of the capillary, the tip was immersed in electrophoretic gel. A glass capillary filled with a physiological solution (containing 6,4 mM KCl, 34 mM glucose, 10 mM Hepes, 12 mM MgCl₂, 1 mM CaCl₂, 12 mM NaCl; pH=6,5) inserted in the base of the antenna served as reference electrode. Recordings were performed exclusively on pheromone-specific sensilla.

Signals were detected by a Axon Preamplifier (x0,1 LU), and conducted to a computer via an Axoclamp 2B Amplifier and an IDAC 2000 Amplifier. Recordings were performed using Autospike 3.2.

B2.3. Stimuli

All stimuli were presented using filter papers in glass pipettes. The species-specific sex pheromone blend (B) (Picimbon et al. 1997, Gemenio & Haynes 1998) was used in a single dose of 10 ng. Heptanal (H) was used in 4 doses, 1 µg, 10 µg, 100 µg and 1000 µg. Mixtures of the pheromone blend and each dose of heptanal (HB) were used to assess the effect of plant odours on the peripheral olfaction of sex pheromone. Doses of hexane (10 µl) and mineral oil (10 µl) were used as controls, as were blank filter papers. Stimuli were presented for 0.5 s using a Syntech Stimulus Controller CS-55. Between each stimulation, the sensilla were given 2 minutes to recover. Odour-laden air was extracted by a ventilation system.

B2.4. Data treatment and statistical analysis

Recordings of 40 s were performed. Exclusively recordings from sensilla responding in the beginning and the end of the experiment were analyzed. Only action potentials (APs) occuring in the excitatory phase were taken into account. To assess these, they were counted for 0.3 s starting 0.2 s after the stimulation as most sensilla were observed to respond by this time.

Data was processed to reduce effects of high heptanal doses by computing the average number of APs for all heptanal doses, as well as the average number of APs for all mixture

doses. Statistical methods revealed no difference between hexane, mineral oil, and blank filter paper as stimuli. These groups were thus pooled as controls (C).

Thus processed data was tested for homogeneity of variance and normal distribution. Since both were not found, nonparametric methods were applied (Kruskal Wallis H-test and *a posteriori* Multiple Comparisons).

B3. Results

Overall, recordings from over 35 sensilla were performed. However, only 14 of these were regarded to fit the requirements for statistical analysis.

The average response to sex pheromone, heptanal, and to the mixture were 19, 3, and 5 APs respectively. Pheromone-specific sensilla hardly responded to any of the control substances.

The response to sex pheromone was significantly higher than to any other substance/mixture tested (KW-H-test; $H_{(3, n=14)}=40,65$; $p<0,001$) (Fig. B1). Combination of heptanal and sex pheromone led to a significant reduction in the response compared to the response to sex pheromone alone (KW-H-test; $H_{(3, n=14)}=40,65$; $p<0,001$). Response intensity to H and HB did not differ statistically. The response to HB was significantly higher than to C (KW-H-test; $H_{(3, n=14)}=40,65$; $p<0,05$), the response intensity to C and H did not differ.

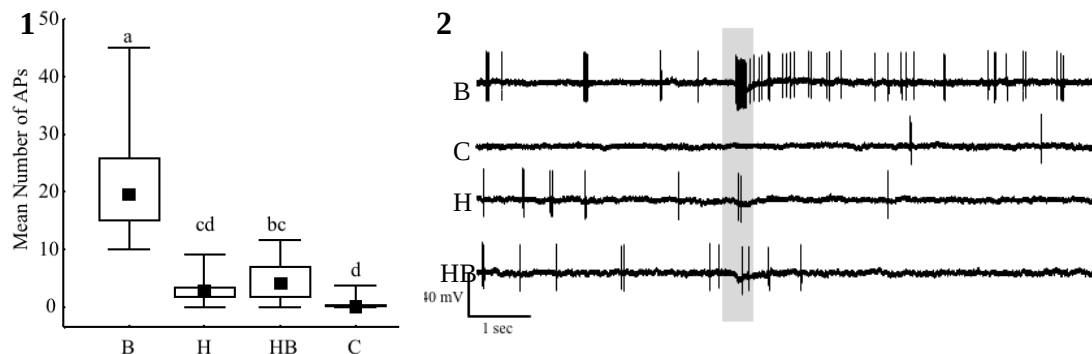


Figure B1. Response of single sensilla to different stimuli. **1:** Results of the statistical methods employed. Black boxes indicate medians, surrounding boxes quartiles, whiskers extreme values. Response to sex-pheromone is never smaller than 10 APs. Response to the sex-pheromone blend is the highest, all other tested stimuli produce significantly weaker responses. Lower cases letters indicate significant differences. For abbreviations see text. **2:** Typical response pattern of a sensillum to (from top down) sex-pheromone, controls, heptanal and mixture of pheromone and heptanal. The gray bar indicates the duration of the stimulation.

The results suggest that the addition of plant odours inhibits the ORN response to sex pheromone in the male *A. ipsilon*. This confirms previous results found in *S. littoralis* (Party et al. 2009).

Comment: As a part of a research project on the effect of plant odours on the behavioural and neuronal response to sex pheromone of *A. ipsilon* males, I performed a series of single sensillum recordings. This side study came in addition to the planned projects of my Diploma thesis.

6. References

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Zusammenfassung

Viele Tiere benutzen ihren Geruchssinn zum Auffinden von Nahrung und Geschlechtspartnern. Bei vielen Nachtfaltern ist dazu ein besonders effektives System der Partnerfindung evolviert, bei dem das Weibchen Sexualpheromone produziert und aussendet, die von den Männchen besonders gut wahrgenommen werden können. Die Sensibilität der Männchen für die arteigenen Sexualpheromone ist so hoch, dass eine Partnerfindung über lange Distanzen möglich ist.

Die männliche Ypsiloneule, *Agrotis ipsilon*, erlebt jedoch nach der Paarung eine Periode von verringerter Sensibilität auf das artspezifische Sexualpheromon, die Post-Ejakulations Intervall (PEI) genannt wird. Während dieser Phase werden die männlichen Falter nicht von Sexualpheromon bzw. den pheromonaussendenden Weibchen angezogen. Das PEI dauert bis zur nächsten nächtlichen Aktivitätsperiode, von diesem Zeitpunkt an ist die ursprüngliche Sensibilität vollständig wiederhergestellt. Dieses Pausieren des Sexualverhaltens erlaubt den verpaarten Männchen für die Fortpflanzung wichtige Proteine in den Akzessorischen Sexualdrüsen (ASD) zu sammeln, die bei der vorherigen Paarung vollständig entleert wurden. Damit verhindern die Männchen Verpaarungen mit geringer Befruchtungswahrscheinlichkeit. Diese Plastizität im Sexualverhalten wird maßgeblich bestimmt durch eine verringerte Sensibilität auf Sexualpheromon der Projektionsneurone (PN) in den primären olfaktorischen Zentren, den Antennalloben (AL). Das beobachtete PEI könnte durch Veränderungen in der Produktion neuroregulatorischer Peptide, biogener Amine oder Ecdysteroidhormone reguliert werden.

Auch wenn bis dato keine Mechanismen bekannt sind, die das PEI bei Insektenmännchen auslösen, so ist doch bei Weibchen vieler Insektenarten (zum Beispiel aus den Ordnungen Diptera oder Lepidoptera) eine Refraktärperiode bekannt, die durch eine vorhergegangene Paarung ausgelöst wird. Dabei werden vom Männchen Peptide wie das Sex-Peptid (SP) übertragen, die von den männlichen ASD gebildet werden und im weiblichen Organismus über spezifische Rezeptoren wirken. Es ist möglich, dass im männlichen Organismus ähnliche Peptide das PEI auslösen.

Um das Ursprungsorgan des männlichen PEI zu finden, wurden die Effekte von Injektionen mit Gehirnen oder ASD auf das Verhalten von unverpaarten und verpaarten männlichen Faltern in Windtunnelexperimenten beobachtet.

Wesentlich wurden zwei Hypothesen verfolgt: i) unverpaarte Männchen produzieren eine Substanz die ihre Empfindlichkeit für das weibliche Pheromon aufrechterhält, und die nach der Paarung nicht mehr oder in verringertem Maß produziert wird & ii) verpaarte Männchen

erzeugen auf Grund der Paarung eine Substanz die das PEI induziert. Zusätzlich wurden die möglicherweise unterschiedlichen Peptidprofile in den Gehirnen unverpaarter und verpaarter Männchen mittels MALDI-TOF Massenspektrometrie verglichen.

Die Ergebnisse zeigen deutlich, dass das Verhalten verpaarter Männchen nicht durch die Injektion von Geweben unverpaarter Männchen beeinflussbar ist. Im Gegensatz dazu ist es durchaus möglich mittels der Injektion von ASD, aber nicht Gehirnen, verpaarter Männchen eine Reduktion des Verhaltens unverpaarter Männchen zu erreichen.

Weiters zeigen die vorläufigen Ergebnisse der MALDI-TOF Massenspektrometrie keine drastischen Veränderungen im Peptidprofil nach der Paarung.

Demnach kann geschlossen werden dass die männlichen ASD Substanzen produzieren, die nicht nur das Verhalten des Weibchens verändern, sondern auch auf das Männchen selbst einwirken. Nachdem verpaarte Männchen Verhaltensänderungen zeigen die denen verpaarter Weibchen ähneln, wird angenommen, dass ein Peptid ähnlich dem SP das männliche PEI auslöst. Alternativ dazu könnte die Steuerung des PEI durch Dopamin (DA) oder das Insektenhormon 20-Hydroxyecdysol erfolgen, da beide Substanzen nachgewiesenermaßen von großer Bedeutung für die männliche Sexualität von Vertebraten und Insekten sind.

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