Recognition and Modulation of Olfactory Signals in the Noctuid Moth *Spodoptera littoralis*

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Cover: Female and male *Spodoptera littoralis*
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Abstract

Food, mates and host plants are essential resources for plant-feeding insects. Optimal resource localization that fulfils the physiological need and reproductive goals of an insect is of utmost importance. Insects, like most animals, rely largely on the sense of smell to locate and evaluate potential resources that enhance their reproductive fitness. Confronted with a multitude of stimuli ranging from food to conspecifics, insects execute behavioural strategies that are strongly modulated by internal physiological factors such as hunger and reproductive status. In this thesis, I address how an insect's reproductive state modulates olfactory perception and ultimately behaviour towards adult food, female sex pheromone and larval host plant odours.

The cotton leafworm *Spodoptera littoralis* is an appropriate species for studying modulation of olfactory perception and behaviour, since the chemical ecology and olfactory physiology of mate finding and host seeking is being thoroughly studied. Here, I show that mating in female *S. littoralis* causes transient reduction in the sexual receptivity, and a reduction in longevity. Mating induces physiological changes that strongly influence olfactory coding and preference in males and females that match their current physiological need. Following mating, female *S. littoralis* switches olfactory preference from adult food to egg-laying cues. Unmated female *S. littoralis* are highly attracted to lilac flowers (*Syringa vulgaris*). After mating, females switch their olfactory preference to the host plant cotton (*Gossypium hirsutum*). Remarkably, the behavioural switch from floral to green odours is also mirrored in the primary olfactory centre, the antennal lobe (AL). Further, I have identified odorants from cotton that elicit robust upwind flight responses in gravid female *S. littoralis*.

In male *S. littoralis*, mating transiently abolishes attraction to pheromone-releasing females and cotton volatiles that signal larval habitat and mating sites. This behavioural modulation is reflected in the peripheral and central olfactory system, the antenna and the AL. In contrast, behavioural and neuronal responses to lilac flowers that signal an adult food source are not influenced by mating status. These findings provide an excellent substrate to examine how neuronal circuits integrate external sensory information with physiological state to shape behaviour.

Keywords: Spodoptera littoralis, *Spodoptera litura*, olfaction, modulation, mating, antennal lobe, wind tunnel, electrophysiology, optical imaging

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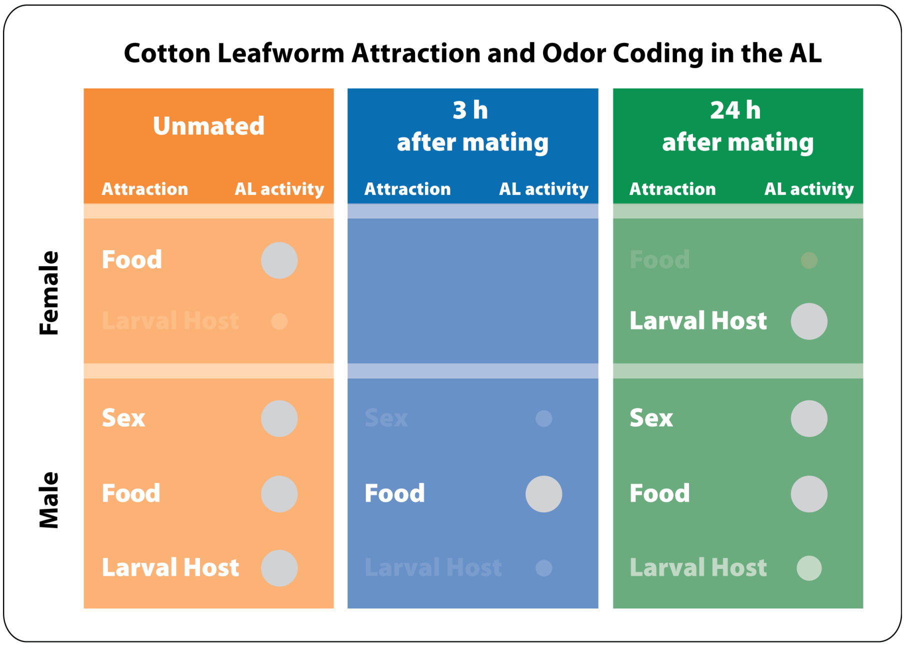
Graphical Abstract



Mating-dependent upwind attraction and odour coding in a noctuid moth. Flight attraction of unmated and mated (3 h and 24 h after mating) males and females of *Spodoptera littoralis* towards sex (calling females), food (lilac flowers), and larval host (cotton plant) odorants in a flight tunnel. Circles indicate antennal lobe activity (odour intensity coding); large and small circles indicate strong and weak antennal lobe responses, respectively.

Dedication

To my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I A. M. Saveer, P. G. Becher, G. Birgersson, B. S. Hansson, P. Witzgall and M. Bengtsson. Mate recognition and reproductive isolation between the sibling species *Spodoptera littoralis* and *S. litura* (submitted).

II A. M. Saveer, S. H. Kromann, G. Birgersson, M. Bengtsson, T. Lindblom, A. Balkenius, B. S. Hansson, P. Witzgall, P. G. Becher and R. Ignell (2012). Floral to green: mating switches moth olfactory coding and preference. *Proceedings of the Royal Society B* 279(1737), 2314-2322.

III S. H. Kromann, A. M. Saveer, M. Binyameen, M. Bengtsson, G. Birgersson, B. S. Hansson, F. Schlyter, P. Witzgall, R. Ignell and P. G. Becher. Concurrent modulation of olfactory responses to sex and host plant cues in a male moth (manuscript).

IV A. M. Saveer, P. G. Becher, G. Birgersson, B. S. Hansson, M. Bengtsson and P. Witzgall. Upwind flight attraction of *Spodoptera littoralis* female moths to natural and synthetic cotton volatiles (manuscript).

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The contribution of Ahmed M. Saveer to the papers included in this thesis was as follows:

I Designed, conducted and analysed experiments and wrote the manuscript together with co-authors.

II Designed, conducted and analysed all behavioural, analytical (headspace sampling, GC-MS analysis), electrophysiological (GC-EAD, EAG) experiments and wrote the paper together with co-authors. AM Saveer and ASH Kromann share joint first authorship.

III Designed, conducted and analysed all behavioural, analytical (headspace sampling, GC-MS analysis) and electrophysiological (GC-EAD) experiments and contributed to the preparation of manuscript. ASH Kromann and AM Saveer share joint first authorship.

IV Designed, conducted and analysed all experiments and wrote the manuscript together with co-authors.

Abbreviations

AL	Antennal lobe
ORN	Olfactory receptor neuron
OR	Odorant receptor
OBP	Odorant-binding protein
GR	Gustatory receptor
IR	Ionotropic receptor
LN	Local interneuron
PN	Projection neuron
MB	Mushroom body
LH	Lateral horn
GC-MS	Gas chromatography-mass spectrometry
GC-EAD	Gas chromatography-electroantennographic detection

1 Objectives

The interplay between an animal's physiological state and external sensory stimuli is the basis for many behavioural decisions which influence reproductive fitness. The objective of this thesis was to investigate olfactory-mediated resource detection which is critical for reproductive success and how this process is modulated by the internal physiological state in *S. littoralis*. The study has been carried out in three consecutive steps. First, the effect of mating on the lifespan and reproductive fitness of male and female *S. littoralis* was investigated. Secondly, the question how mating state modulates the olfactory-driven innate behaviour towards food (*Syringa vulgaris*), sex (calling female *S. littoralis*) and host plant (*Gossypium hirsutum*) was addressed. And finally, key potential odorants that mediate the attraction of *S. littoralis* females to cotton, *G. hirsutum* were identified.

2 Introduction

2.1 Insects: From beneficial to harmful disease vectors

Insects are most abundant with respect to the number of species and biomass: 80% of the known animal species are insects (Hill, 1997). One of the fundamental reasons for the evolutionary success of insects is their adaptability to different habitats. Many insects directly or indirectly are beneficial to humans. Insects are necessary for pollination of agricultural crops, for the production of honey and silk, as animal food, and as biocontrol agents. On the other hand, insects are also pests that cause tremendous economic loss to humans. Insects are also disease vectors causing millions of deaths both in humans and livestock (Hill, 1997).

Most insects live on green plants, which serve as the primary source of energy for heterotrophic organisms. Certainly, insects and plants are inextricably linked, and the evolution of plant-insect association has been mediated to a large extent by plant chemistry for the majority of insect groups (Schoonhoven *et al.*, 2005). The role of secondary plant chemistry has been the subject of many discussions on the evolution of the host range of phytophagous insects, on speciation and also on the development of sustainable crop protection strategies.

2.2 Resource localization: Physiological state and behavioural decisions

Localization of resources such as food, mate, and host plants is an essential component of life history traits that are critical for the growth, development, and reproduction of animals, including insects (Bell, 1990). Resources are distributed heterogeneously across time and space. Locating optimal resources entails costs associated with search time, risk of predation and other environmental factors (Browne, 1993; Bell, 1990). Furthermore, the resource requirement by insects varies according to the developmental stage, sex, and endogenous factors like nutritional and mating state (Browne, 1993). For appropriate resource-oriented behaviour, insects need to keep track of current physiological state and external sensory stimuli relevant to the required behaviours (Mowrey & Portman, 2012; Browne, 1993).

In most organisms, including insects, physiology and behaviour are inextricably linked and are regulated by a number of factors. Mating is one such factor that triggers profound physiological and behavioural changes (Anton *et al.*, 2007; Gillott, 2003; Chen, 1984). The common fruit fly *Drosophila melanogaster*, a model species in olfaction research, exhibits a repertoire of mating-induced changes such as reduced female receptivity, stimulation of oviposition, reduction in longevity, modulation of feeding behaviour, and decrease in siesta sleep (Avila *et al.*, 2011; Chapman *et al.*, 2003). Male and female receptivity in butterflies and moths is also affected by mating (Wedell, 2005). In addition, mating reduces immunity (vulnerable to infection) in the mealworm beetle *Tenebrio molitor* (Rolff & Siva-Jothy, 2002) and reduces flight activity in the honey bee *Apis mellifera* (Kocher *et al.*, 2010). In lepidopteran and dipteran species, the transient or permanent post-mating changes are largely due to the transfer of male sperm and other seminal fluid proteins (SFPs) during copulation (Wedell, 2005; Gillott, 2003; Jin & Gong, 2001; Wolfner, 1997; Bali *et al.*, 1996; Chen *et al.*, 1988; Chen, 1984). In *D. melanogaster*, male accessory gland extract contains a sex peptide that reduces receptivity and enhances the egg production when injected into unmated females (Chapman *et al.*, 2003). In moths, other factors such as juvenile hormone and ecdysteriods play a prominent role in such physiological and behavioural switches (Wedell, 2005). In the tobacco budworm *Heliothis virescens* males transfer juvenile hormone to the females during copulation that stimulate egg maturation (Ramaswamy *et al.*, 2000; Park *et al.*, 1998).

Mating induced physiological changes strongly influence behavioural decisions that further modulate the neural responses to auditory, olfactory and tactile sensory stimuli. This sensory gating allows insects to execute appropriate behaviour from a repertoire of possible responses (Mowrey & Portman, 2012). In the Mediterranean fruit fly *Ceratitis capitata*, mated females switch their odour preference from male pheromone to a host plant odour (Jang, 1995). Females of *D. melanogaster* also undergo a similar dietary switch following mating (Vargas *et al.*, 2010). In the male cutworm *Agrotis ipsilon*, mating induces transient behavioural and central nervous system inhibition to female sex pheromone (Gadenne *et al.*, 2001). Mating also switches olfactory coding and behavioural preference in female *S. littoralis*

(Saveer *et al.*, 2012). Taken together, this suggests that an animal's behavioural decisions are strongly modulated by the internal physiological state allowing them to execute appropriate behaviours at the right time and right place to enhance their reproductive fitness.

2.3 Chemical communication

Animals rely on their sensory systems to perceive the environment in which they live. The sensory systems can be classified according to the type of stimulus they register; sound (acoustic), light (vision), and chemicals (smell and taste) and their relative importance varies from one species to another (Dangles *et al.*, 2009). The chemical senses are phylogenetically ancient and they are shared by all organisms, from bacteria to mammals (Wyatt, 2003). External chemical information is vital to locate and evaluate food, host, shelter, mates, and breeding substrates as well as the location of predators. From a physiochemical point of view, chemical substances differ in shape, size, functional group, carbon chain length, volatility, polarity etc. Natural chemical stimuli typically are mixtures of several compounds that vary in composition, proportion and aerial concentration. An animal's olfactory system can detect such airborne volatile blends of chemicals and distinguish between them with high precision.

Chemical signals that are used for communication are termed semiochemicals, or sometimes infochemicals. Pheromones are a subclass of semiochemicals, which are used for communication within the same species (intraspecific communication) and are widely used by a variety of organisms, from moths to elephants (Wyatt, 2003). Semiochemicals that act between individuals of different species are called allelochemicals and are further classified based on the cost and benefits to the receiver and the signaller (Nordlund & Lewis, 1976). Allelochemicals that benefit the receiver and affect the signaller, such as plant volatiles mediating insect attraction, feeding and oviposition, are termed kairomones. Allelochemicals that benefit the signaller and negatively affect the receiver, such as plant volatiles that act as repellent and deterrent against herbivores, are termed allomones. Finally, allelochemicals beneficial to both signaller and receiver, such as flower volatiles that attract pollinators, are termed synomones.

2.4 Insect pheromone communication

Sex pheromones are important chemical signals for intraspecific recognition across many taxonomic groups and play a crucial role in speciation events.

Recognition of compatible mating partners is an important prerequisite for reproductive success. Among other functions, sex pheromones also convey information about sex, physiological status and age (Wyatt, 2003). Usually sex pheromones are blends of compounds, typically they consist of one major component and several minor components that vary in identity or ratio between species. In moths, long-range attraction between males and females is mediated by female-produced sex pheromone. In addition, males of some moth species such as the European corn borer, *Ostrinia nubilalis* release pheromone through hair pencils that act as a short-range signal (Lassance & Löfstedt, 2009; Phelan & Baker, 1987). Genetically compatible species recognition occurs mainly due to the whole blend emitted by the female, and not to the major component alone (Linn & Roelofs, 1989). Presence of minor components gives the signal its specificity as shown in the oriental fruit moth, *Grapholitha molesta* (Linn *et al.*, 1987). The role of sex pheromones in the evolution of reproductive isolation has, for example, been shown in the small ermine moths, *Yponomeutidae* (Löfstedt *et al.*, 1991). Other factors such as allochronic separation of female moth calling behaviour, host plant, and physiological factors may also play an important role and enhance the specificity of the pheromone communication channel (Groot *et al.*, 2010).

2.5 Insect food and host location

To increase the reproductive fitness in terms of longevity and fecundity, insects need to efficiently locate suitable food and host sites (Wenninger & Landolt, 2011; Song *et al.*, 2007). Behavioural and electrophysiological studies on a wide range of phytophagous insects have supported the notion that insects use blends of volatile compounds to locate their host plants (Riffell *et al.*, 2009; Tasin *et al.*, 2006; Bruce *et al.*, 2005), suggesting that insects have sufficient specific olfactory receptors (ORs) to detect ubiquitous plant volatiles. Indeed, there are numerous examples showing insect olfactory receptor neurons (ORNs) specifically tuned not only to the pheromone but also to general plant compounds (Bruce *et al.*, 2005; Stensmyr *et al.*, 2001; Hansson *et al.*, 1999). If the majority of peripheral receptors of phytophagous insects detects compounds that are not unique to their host plants, then the ratio of volatiles emitted by the plant becomes a vital component of the olfactory signal, so-called 'blend-specific odour recognition' (Bruce *et al.*, 2005). In this case specificity would depend on the particular ratio of components rather than one single compound. In addition, any distortion in the natural ratio would result in disruption of olfactory orientation to the host plant. Many more studies describe that phytophagous insects use blend composition to locate host plants.

For example, a 3-component blend of grape volatiles, (*E*)-β-caryophyllene, 4,8-dimethyl-1,3(*E*),7-nonatriene and (*E*)-β-farnesene in a 100:78:9 ratio at remarkably low concentration attracts the female grapevine moth, *Lobesia botrana*, in a laboratory flight tunnel study. Any distortion in the 3-component blend abolishes attraction (Tasin *et al.*, 2007; Tasin *et al.*, 2006). A 3-component blend of peach volatile compounds was attractive to the mated female oriental fruit moth, *Cydia molesta*. Attraction was abolished when the single individual compounds were tested (Natale *et al.*, 2003). Another behavioural study on *C. molesta* demonstrates that a 5-component synthetic mixture was as attractive to female moths as the natural blend, whereas none of the components were attractive when tested separately. When tested physiologically, the behavioural active compounds showed unique synergistic pattern in the AL (Piñero *et al.*, 2008; Piñero & Dorn, 2007).

2.6 Insect olfactory system: An overview

2.6.1 Insect peripheral anatomy

The principle insect olfactory organ is the antenna. Apart from the antenna, insects also use maxillary palps in odour detection. Insect antennae vary in shape and are divided into three parts; scape (basal segment attached to the head capsule), pedicel (attached to the scape with elastic membrane), and flagellum (main part of the antenna). The flagellum carries most of the sensilla, which show a wide variety of shapes and structures (Keil, 1999). An olfactory sensillum houses the dendrites of one to several bipolar ORNs surrounded by auxiliary cells. The dendrites of the ORNs are surrounded by sensory lymph that is produced by support cells at the base of the sensillum (Keil, 1999). Six different types of olfactory sensilla identified in female *S. littoralis* are: basiconic, trichoid (long and short), coeloconica, auricillic and grooved peg (Binyameen *et al.*, 2012).

2.6.2 Odorant receptors

Odour perception begins when odour molecules are captured by the sensilla and diffuse through narrow pores into the sensillum lymph. Here, odour molecules bind to the highly abundant odorant-binding proteins (OBPs) that transport them to the odorant receptors (ORs) on the dendritic membrane of olfactory receptor neurons (ORNs) (Hildebrand & Shepherd, 1997). Insect ORs along with gustatory receptors (GRs) and ionotropic receptors (IRs) together constitute the insect chemosensory receptors (Benton *et al.*, 2009; Clyne *et al.*, 2000; Vosshall *et al.*, 1999). These ORs, IRs and GRs are primarily responsible for detecting odorants and tastants, respectively. However, in *D. melanogaster* GRs are also expressed in ORNs that can detect carbon dioxide (CO_2) (Jones *et al*., 2007). Insect ORs constitute a highly divergent gene family that does not share sequence similarity with the vertebrate and nematode OR family (Bargmann, 2006). Insect ORs have a seven transmembrane-domain with an inverted transmembrane topology, with an intracellular N-terminus and an extracellular C-terminus (Benton *et al*., 2006). Furthermore, insect ORs function as heterodimer complex and typically consist of a single ligand-binding OR and an ubiquitously expressed OR coreceptor (Orco) (Vosshall & Hansson, 2011; Larsson *et al*., 2004). Studies have shown that the OR-Orco heteromer functions as an odorant-gated ion channel and also take part in signal transduction (Sato *et al*., 2008; Wicher *et al*., 2008).

2.6.3 Odour coding

All olfactory stimuli consist of many single odorants that combine to form complex odour mixtures. Thus, the olfactory system of insects must code for both specific and generic odours. This is mainly achieved by the large OR repertoire family expressed in the ORNs. Although ORNs expressing the same OR are distributed over the antenna, they converge at the same region of the AL, in subunits called glomeruli (Tolbert & Hildebrand, 1981). The functional characterization of the OR repertoire studies from *D. melanogaster* and African malaria mosquito *Anopheles gambiae* reveals several fundamental principles of odour coding. The ORs display a varying degree of specificity, with individual odorants being perceived by subsets of receptors. Some ORs are narrowly tuned to a few odorants, whereas some receptors are broadly tuned, being activated by large number of odorants (Carey *et al*., 2010; Hallem *et al*., 2004). In a combinatorial code, most odour stimuli elicit responses in more than one glomerulus. Thus, activity across AL glomeruli corresponds to the activity across receptor types. Taken together, the above mentioned studies strongly support the combinatorial model of odour coding in the insect olfactory system as shown in vertebrates. In addition, insects also use labelled line coding, where an odorant activates a single narrowly tuned ORN class. The best-studied specialist ORNs are those responding to pheromones. In moths, female produced sex pheromone activates selectively tuned neurons present in the male antenna and their cognate glomerulus (Christensen & Hildebrand, 1987). A similar class of narrowly tuned ORNs was also found in *D. melanogaster*, where they were activated by the pheromone *cis*-vaccenyl acetate (cVA) and CO_2, respectively (Datta *et al*., 2008; Jones *et al*., 2007).

2.6.4 Insect antennal lobe

There are two types of neurons in the AL: local interneurons (LNs) and projection neurons (PNs; Figure 1). LNs do not form connections outside the AL and receive input from both ORNs and PNs. LNs can be inhibitory or excitatory, releasing γ-aminobutyric acid (GABA) or probably acetylcholine (Masse *et al.*, 2009), respectively. Both excitatory and inhibitory neurons form extensive connections throughout the AL and make inter- and intra-glomerular connections. PNs are the output neurons of the AL, which convey odour information to higher brain centers in the protocerebrum, the lateral horn (LH) and the mushroom body (MB; Figure 1). In *D. melanogaster* and *A. mellifera*, there are two types of PNs: uniglomerular and multiglomerular. Uniglomerular PNs have input synapses within the AL in only one glomerulus, and send their axons to MB and LH. Multiglomerular PNs branch in many glomeruli, and extend their axons to the LH but bypass the MB (Galizia & Szyszka, 2008). Most of the PNs are cholinergic but a small number of them are known to be GABAergic (Masse *et al.*, 2009).

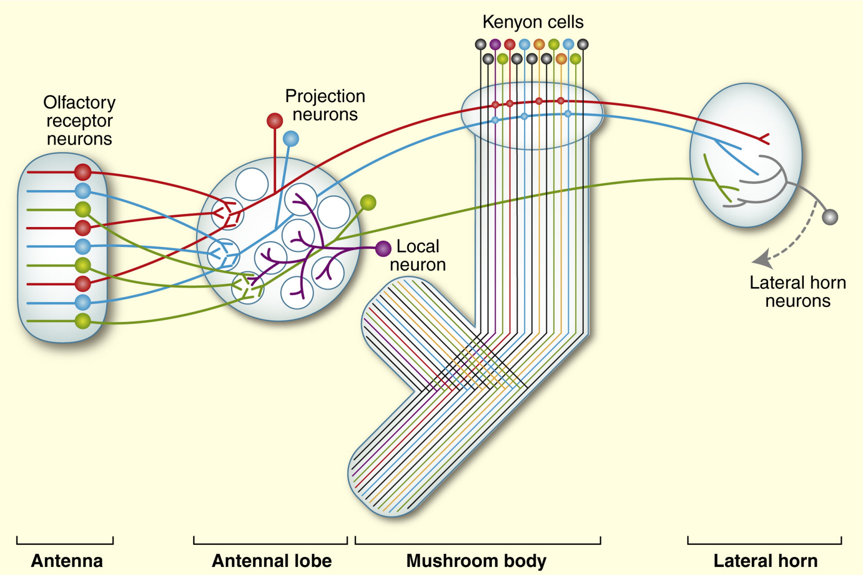

Figure 1. Overview of insect olfactory system. Olfactory receptor neurons (ORNs) in the antennae expressing the same odorant receptor (same colour) send axons to the same individual glomerulus in the antennal lobe. In the antennal lobe, ORNs form synaptic contacts with projection neurons (PNs) and local interneurons (LNs). PNs either send their axons directly to the lateral horn (green axon), or indirectly through Kenyon cells of the mushroom body and then to the lateral horn red and blue axons). Reproduced from (Masse *et al.*, 2009) with permission from Elsevier.

3 The model system

The cotton leafworm *S. littoralis* (Boisduval) (Lepidoptera, Noctuidae) is an appropriate model organism for investigations of the neurophysiological basis of odour-mediated behaviour, since the chemical ecology and olfactory physiology of mate finding and host seeking has been thoroughly studied (Binyameen *et al.*, 2012; Anton *et al.*, 2011; Anderson & Alborn, 1999; Anderson *et al.*, 1995; Anton & Hansson, 1995; Anderson *et al.*, 1993). *S. littoralis* is highly polyphagous and a highly destructive agricultural pest on a range of crops (Nagoshi *et al.*, 2011; Brown & Dewhurst, 1975; Salama *et al.*, 1971). Having the capacity for extensive migration *S. littoralis* constitutes a great invasive threat and therefore is an important model system to study, also from an ecological and pest management perspective. Geographically *S. littoralis* is distributed across Africa, southern Europe and the Middle East regions (Staneva, 2009).

Male and female *S. littoralis* are active during early and mid-scotophase. The calling activity (pheromone release) of female *S. littoralis* increases gradually with most females calling during the mid scotophase, thereafter calling decreases sharply (data not shown). Male flight activity was highest during early and mid scotophase. Female *S. littoralis* sex pheromone shows considerable variation across geographical regions (Munoz *et al.*, 2008). The female pheromone gland of moths from an Egyptian population consists of a blend of 11-components (Table 1). Males were highly sensitive and attracted to the female pheromone gland extracts in laboratory wind tunnels and field (Dunkelblum *et al.*, 1982; Kehat *et al.*, 1976; Paper 1). Male and female *S. littoralis* are polyandrous i.e. they mate multiple times during their lifetime (Sadek, 2001; Kehat & Gordon, 1975). Female *S. littoralis* initiates batch wise egg-laying 2-3 hours after mating. Each female lays 7-8 batches of eggs that take 4-5 days to hatch. The larvae pass through six instars and then pupate. The longevity of unmated adults is about 12-14 days.

The oriental leafworm or tobacco cutworm *Spodoptera litura* (Fabricius) (Lepidoptera, Noctuidae) is another most important agricultural pest species in the genus *Spodoptera*. *S. litura* is also polyphagous and feeds on a range of economically important crops. Geographically, *S. litura* occurs in the Middle East, throughout the Indian subcontinent and South-East Asia, Australia and the Pacific Islands (International, 1967). The border between the geographic distribution of *S. littoralis* and *S. litura* is in Pakistan and Southern Iran. Although there are no reports showing their overlap, the two species might meet at a narrow zone in the southern provinces of Iran (personal communication). The *S. litura* female pheromone consists of a blend of four components (Paper 1) and all of them were also found in *S. littoralis* with minor differences in relative amounts.

Compound	Cyprus [a]	Kenya [b]	Israel [c]	Spain [d]	Spain [e]	Spain [f]	Egypt [g]
14:OAc	28	–	–	1	9[†]	1	1
Z9-14:OAc	3	–	46	13	14	11	14
E11-14:OAc	14	–	9	10	10	11	9
Z11-14:OAc	–	–	7	5	9[†]	7	6
Z11-16:OAc [*]	–	–	–	–	–	–	4
Z9,E11-14:OAc	55	95	33	71	56	57	47
Z9,Z11-14:OAc [*]	–	–	4	–	–	–	1
E9,Z11-14:OAc [*]	–	–	–	–	–	–	3
E9,E11-14:OAc [*]	–	–	–	–	–	–	3
Z9,E12-14:OAc	–	5	0.5-1	–	–	–	2
E10,E12-14:OAc	–	–	–	–	11	14	10

[*] Compounds identified for the first time in *S. littoralis*.; [†] Z11-14:OAc + 14:OAc

Table 1. *Literature overview of pheromone gland composition in Spodoptera littoralis females from different geographical regions. (a) (Nesbitt et al.,* 1973*) (b) (Tamaki & Yushima,* 1974*) (c) (Dunkelblum et al.,* 1982*) (d) (Martinez et al.,* 1990*) (e) (Navarro et al.,* 1997*) (f) (Munoz et al.,* 2008*) (g) present study (numbers in the table represents the relative amounts).*

4 Summary of results

4.1 Mate recognition and post-mating physiological changes in closely related sibling species *S. littoralis* and *S. litura* (Paper – I)

Reproductive success that leads to viable and fertile offspring is the culmination of compatible mate recognition and successful fertilization. Here we show that the females of *S. littoralis*, *S. litura* and hybrids produce sex pheromone that differs both in quantity and quality. Four compounds *Z*9-14:OAc, *Z*9,*E*11-14:OAc (major component), *Z*9,*E*12-14:OAc and *E*10,*E*12-14:OAc co-occur in female pheromone glands of both *S. littoralis*, *S. litura* and their hybrids. Previous work shows a geographical variation in the *S. littoralis* female pheromone composition (Munoz *et al.*, 2008). We identified in *S. littoralis* originating from Egypt for the first time all three geometric isomers of the main pheromone compound, *E*,*Z*-, *E*,*E* and *Z*9,*Z*11-14:OAc together with three monoenic acetates, which were not found in *S. litura* female glands. Despite the fact that females of *S. littoralis* and *S. litura* share common major and minor pheromone components, males of *S. littoralis* discriminate conspecific from heterospecific calling females in a dual-choice test in a wind tunnel bioassay. However, in a no-choice assay, as many *S. littoralis* and *S. litura* males responded to conspecific and heterospecific calling females (Figure 2).

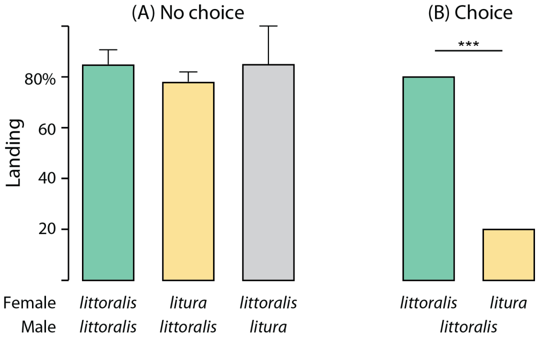

Figure 2. Upwind flight attraction and landing of (A) male *Spodoptera littoralis* (n = 50) and *S. litura* (n = 20) at conspecific and heterospecific calling females in a no-choice wind tunnel test. (B) Male *S. littoralis* attraction to conspecific and heterospecific calling females (n = 50, p < 0.0001, exact binomial test).

Conspecific and heterospecific matings between males and females of both species revealed an asymmetry: matings occur between *S. littoralis* females and *S. litura* males, while *S. litura* females do not mate with *S. littoralis* males. The mating duration and mating frequency of three possible mate combinations were comparable (Figure 3). Post-mating reproductive fitness was much higher when *S. littoralis* and *S. litura* females mated with conspecific males, lead to 90 – 95% egg fecundity. Interestingly, the heterospecific matings, *S. littoralis* (female) x *S. litura* (male), dramatically reduced the reproductive fitness (Figure 4). Furthermore, our findings show the effect of mating on the longevity of the female moths. Mating with respective conspecific males significantly reduces the lifespan of both female *S. littoralis* and *S. litura* post-mating. However, the life span of female *S. littoralis* even further reduced after mating with *S. litura* males (Figure 5). Taken together our findings show that mating infers a physiological cost in females. Furthermore, hybrid matings between these closely related species have a negative fitness effect as compared to conspecific matings, emphasizing the importance of compatible mate recognition.

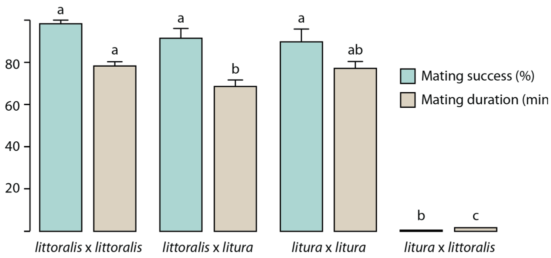

Figure 3. Percentage of successful conspecific and heterospecific *Spodoptera littoralis* and *S. litura* matings (mean ± SE, n = 40 to 50, Chi-square test). Mating duration (min) in conspecific and heterospecific *S. littoralis* and *S. litura* matings. Letters above error bars show significant differences (Kruskall-Wallis test).

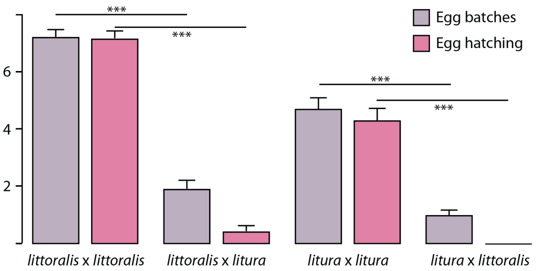

Figure 4. Mean number of egg batches and egg hatching per female, following conspecific and heterospecific matings (mean±SE, n = 40 to 50). Differences are significant at p < 0.0001, according to Mann-Whitney test.

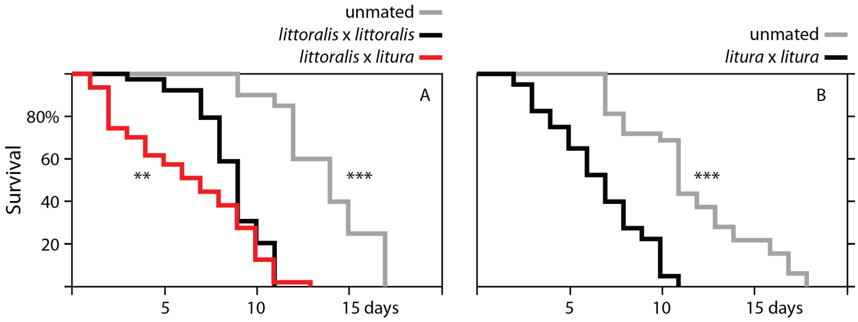

Figure 5. Survival curves of female moths following unmated (grey), conspecific (black) and heterospecific (red) matings, in *Spodoptera littoralis* (A), and *S. litura* (B). Conspecific matings reduced survival significantly at p < 0.0001 (***). Survival after heterospecific mating was further reduced in comparison to conspecific mating at p < 0.001 (**).

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4.2 Mating induced behavioural and olfactory shift in female *S. littoralis* (Paper – II)

Mating triggers dramatic physiological changes in most insects that strongly influence behavioural decisions that are driven by sensory stimuli. Here we show for the first time that a noctuid female moth *S. littoralis* switches the olfactory preference from food to egg-laying cues following mating. Through a series of behavioural experiments, we demonstrate that unmated female *S. littoralis* are highly attracted to nectar-rich flowers of lilac (*S. vulgaris*), whereas females switch attraction to the larval host plant cotton (*G. hirsutum*) for egg-laying, 24 h after mating. Although unmated and mated females initiated flight towards cotton plant and lilac flowers, respectively, significantly fewer moths flew up wind and contacted the source (Figure 6A).

Through gas chromatography-mass spectrometry (GC-MS) and combined GC and electroantennographic detection (GC-EAD), we have identified compounds in lilac and cotton headspace eliciting an antennal response in unmated and mated females. We found 17 active compounds in lilac and six in cotton, with benzaldehyde co-occurring in both headspace samples. Furthermore, calcium imaging recordings were performed using both the headspace extracts of lilac and cotton and synthetic individual GC-EAD active compounds to investigate the olfactory representation of lilac and cotton odours in the primary olfactory centre, the AL. Remarkably, mating significantly modulates the neural input to the AL. Moreover, the behavioural switch is mirrored at the AL level where ensembles of AL glomeruli dedicated to either lilac or cotton odour are selectively up- and downregulated in response to mating (Figure 6B). This olfactory mediated behavioural switch in accordance to the mating state is essential to meat the resource requirement in female *S. littoralis*, since mating induces the fertilization process and egg-laying demands a high quality host.

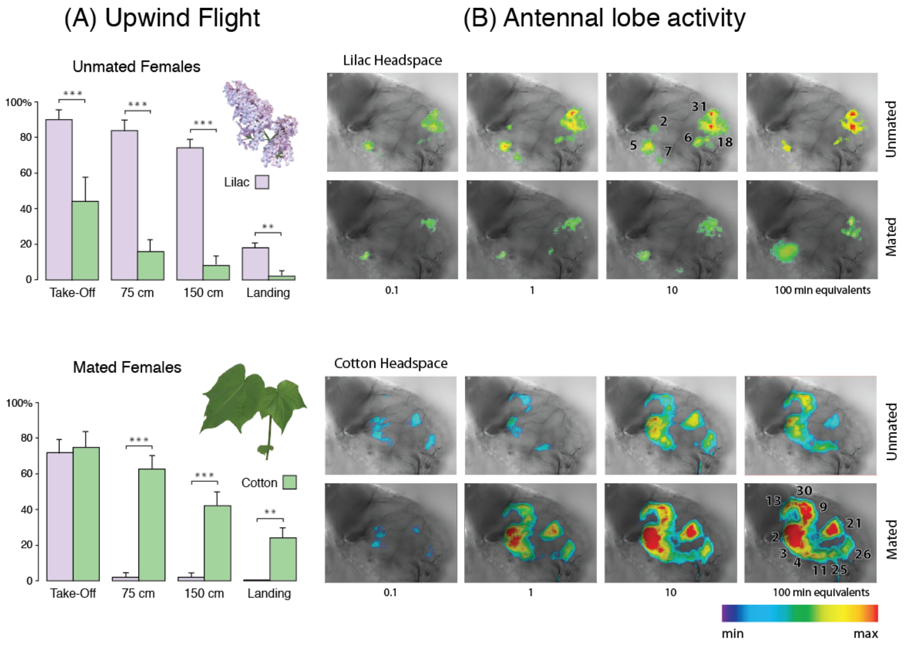


Figure 6. (A) Upwind attraction of unmated and mated cotton leafworm *Spodoptera littoralis* females in a wind tunnel, towards lilac flowers *Syringa vulgaris* and cotton plant *Gossypium hirsutum* (mean ± SEM, n = 50). (B) Calcium response of AL glomeruli of unmated and mated female *S. littoralis* in response to headspace of *S. vulgaris* and *G. hirsutum.*

4.3 Mating induced differential modulation of olfactory responses to host, sex, and food cues in a male moth (Paper – III)

Mating also induces profound physiological and behavioural changes in male *S. littoralis*. Here, we found that male *S. littoralis* differentially modulate odour-driven flight behaviour to females and host plant odours following mating. In a flight tunnel bioassay, unmated males are highly attracted to lilac flowers *S. vulgaris* (food source), calling females *S. littoralis* (mate) and host cotton plant *G. hirsutum* (mating site). Shortly after mating, the behavioural responses to calling females and cotton plants are completely abolished while the attraction to lilac flowers is still maintained. However, the flight response to calling females and cotton plants was either completely or partially restored the following night (Figure 7A). Using GC-MS and GC-EAD, we have identified 14, 6 and 3 antennal active compounds from lilac headspace, cotton headspace and female pheromone gland extracts, respectively. Furthermore, the transient behavioural inhibition to cotton and pheromone odours was reflected at the antennae and the ORN input to the ALs (Figure 7B). Ensembles of AL glomeruli that are tuned to sex pheromone and cotton odours are downregulated immediately after mating and the responses were restored to the normal state in the following night (Figure 7B). Transient post-mating behavioural and central neurons inhibition towards female sex pheromone has been previously reported in males of the noctuid moth *A. ipsilon* (Gadenne *et al.*, 2001). Since male moths probably need time to refill their reproductive glands directly after mating, continued search for females during the same night would not be adaptive. Male attraction to cotton plants may reflect the importance of host plants as mating sites.

Interestingly, neither behavioural nor neuronal responses towards lilac flower odours were affected by mating. This makes biological sense, since nectar is a rich energy source that supplies energy and increases life span (Wenninger & Landolt, 2011). The lack of modulation to the flower odour highlights the differential processing of food and social cues, including pheromones and habitat odorants.

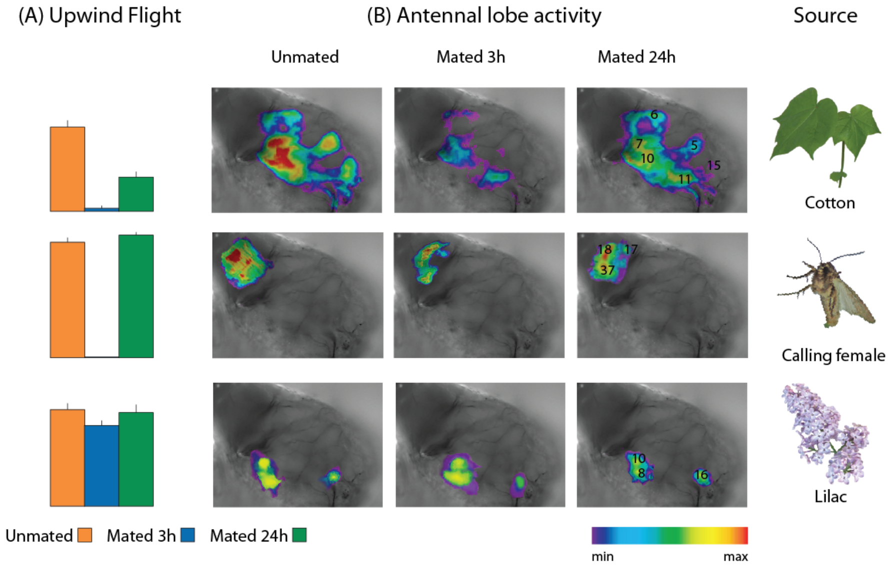


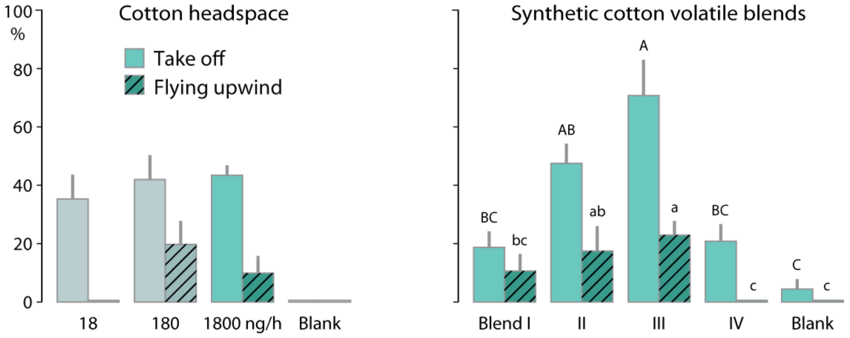
Figure 7. (A) Upwind flight attraction and (B) calcium responses of AL glomeruli of unmated and mated (3 h and 24 h after mating) male *Spodoptera littoralis* towards cotton plant *Gossypium hirsutum*, calling female *S. littoralis*, and lilac flowers *Syringa vulgaris*.

4.4 Flight attraction of *S.littoralis* females to natural and synthetic cotton volatiles (Paper – IV)

Host plant location in a highly complex odorant environment is a daunting task and an essential part of female reproductive success (Bruce *et al.*, 2005). Natural olfactory stimuli generally are blends of several substances that may vary in composition, proportion and aerial concentration. Identification of relevant olfactory stimuli that trigger upwind attraction in gravid female insects while searching for a suitable host plant for egg laying is a current research challenge. After obtaining robust behavioural responses of mated female *S. littoralis* to the host cotton *G. hirsutum*, we here show behavioural responses of mated females to re-vaporized, sprayed natural headspace extracts from cotton. Females displayed robust upwind attraction towards sprayed cotton headspace when released at 180 ng/h and 1800 ng/h of the main compound 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) into the wind tunnel (Figure 8). However, at the lowest dose of 18 ng/h insect did not fly upwind.

To identify the behaviourally relevant odorants from the sprayed cotton headspace, we tested a complete 11-component blend that we have analysed using GC-MS, for comparison with sprayed cotton headspace. The responses were indeed comparable to the complete headspace. In an additive approach, we tested a subset of 4-compounds (nonanal, (*Z*)-3 hexenyl acetate, (*E*)-β-ocimene and (*R*)-(+)-limonene; blend II in Figure 8) that had been shown to evoke a consistent and strong antennal response in *S. littoralis* (Saveer *et al.*, 2012). We found no difference between headspace and 4-component blend. Addition of β-myrcene to blend II further increased upwind flight attraction, making blend III the most attractive synthetic blend. Remarkably, upwind flight of females was strongly inhibited (blend IV) when DMNT was added (Figure 8).

Here we demonstrate that volatile plant compounds mediate the attraction of *S. littoralis* females to their host plant cotton, *G. hirsutum*. Furthermore, behavioural experiments in the wind tunnel confirmed that a subset of few compounds is necessary and sufficient to elicit robust upwind attraction in female *S. littoralis*, supporting previous findings on other moths (Sun *et al.*, 2012; von Arx *et al.*, 2011). A tentative explanation for the antagonistic effect of DMNT on female attraction is that it may signal herbivore damage: DMNT is one of the key components in the volatile blend released from damaged cotton plants (De Moraes *et al.*, 1998; Zakir *et al.,* accepted).



1800 ng/h	DMNT	●			●
1200	Benzaldehyde	●			
850	β-Caryophyllene	●			
830	β-Myrcene	●		●	●
650	Nonanal	●	●	●	●
650	(Z)-3-Hexenyl acetate	●	●	●	●
450	(E)-β-Ocimene	●	●	●	●
230	(R)-(+)-Limonene	●	●	●	●
230	α-Humulene	●			
90	Decanal	●			
30	(R)-(-)-Linalool	●			

Figure 8. Upwind attraction of gravid *Spodoptera littoralis* females to sprayed cotton plant headspace, using a piezoelectric sprayer, at three different doses in a wind tunnel, and to blends of synthetic cotton volatiles that were identified by GC-MS and GC-EAD. Headspace was released at 18, 180 and 1800 ng/h of the main compound DMNT. Synthetic blend composition matched the release rates and proportions found in headspace collections. Sprayed ethanol alone (blank) did not evoke an attraction response. Different letters indicate statistical differences between the blends according to ANOVA followed by Tukey's test (N =3 – 5 batches of 10 moths, mean ± SEM).

5 Conclusion and future perspectives

Noctuid moths rely to a large extent on their sense of smell to successfully locate resources that enhance their reproductive fitness. Although numerous studies have shown attraction of male and female moths to sex pheromones, floral and plant volatiles both in the laboratory and field situations, very few studies have linked these behavioural attractions to internal physiological factors such as hunger and reproductive state. Therefore, the focus of the current study was to investigate the attraction behaviour of the noctuid moth *S. littoralis* towards food, mate and host odours and how such behaviours are modulated by the reproductive state.

In the beginning of the study (paper-I), I have demonstrated that mating causes profound changes in the reproductive physiology and egg-laying behaviour. Conspecific matings resulted in high reproductive fitness by producing large number of fertile eggs and offsrping. Conversely, heterospecific matings showed a dramatic reduction in fertile eggs and also reduced the lifespan of female *S. littoralis*. This suggests differential physiological responses to con- and heterospeciifc matings. This differential cost of matings on the female physiology may be due to non-specific foreign seminal proteins transferred during copulation and complex molecular interactions taking place in the reproductive tract of females. However, more investigation is needed to understand what neuroactive substances are released after mating and how this modulates the sensory apparatus.

In the following part of the study (paper-II and III), I have demonstrated that the reproductive state modulates the olfactory responses of both male and female *S. littoralis* towards distinct olfactory signals. These behaviours match physiological needs such as foraging, mate finding and oviposition. This strongly indicates, that the insect olfactory system is capable of detecting and discriminating different odour mixtures and that it responds differentially according to physiological state. Unmated female moths are strongly attracted to flower related odours for nectar feeding whereas, after mating, females switch the olfactory preference to oviposition substrate cotton (paper-II). Unmated male *S. littoralis* are strongly attracted to female and habitat odors. Shortly after mating, the flight response was completely abolished but was restored to the normal state in the following night. The male response to food odour was not affected by mating (paper-III).

Remarkably, the behavioural responses were also reflected at the primary olfactory centre of the moth brain, the AL. The AL activity of male and female *S. littoralis* is strongly modulated as a result of mating. One of the conundrums in insect olfactory physiology is to understand the mechanisms underlying olfactory modulation. Taking advantage of pheromone-specific ORNs on the male antenna and their cognate glomeruli, this study strongly indicates differential coding pattern of ORs as a result of mating, suggesting modulation at OR level. Further functional characterization of OR repertoires is needed to understand differential coding patterns in the peripheral olfactory system, especially ORs that are tuned to floral and plant odours. The primary representation of odours is further transformed downstream to the AL and modulated before sending the information to higher brain centres. The differential odour intensity coding at the AL as a result of mating suggests the involvement of neuromodulatory substances in the coding of odours. Further studies are needed to understand distribution and the effects of various neuropeptides in the ALs of male and female *S. littoralis*.

Finally, attraction of mated female *S. littoralis* to the plant host cotton is mediated by a blend of volatile compounds (paper-IV). The current finding clearly shows that only a few compounds are necessary and sufficient to evoke robust upwind flight attraction in insect herbivores while searching for a suitable host plant. Future studies aim at understanding how these behaviourally relevant odours are encoded at the primary olfactory centre of the moth brain. Identifying behaviourally meaningful odorants that signify food and host plants will allow to further examine neuronal coding mechanisms and how they are regulated according to physiological state.

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