Neuropeptidergic regulation of mosquito host-seeking behaviour

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Abstract

Mosquitoes are vectors of numerous pathogens that cause human diseases, putting more than half of the world's population at risk. These diseases are transmitted when a mosquito takes a blood meal following the successful seeking of a human host. Host-seeking is highly state dependent and predominantly mediated by olfactory cues. The goal of this thesis is to describe the neuropeptidergic regulation of the state dependent odour-mediated host-seeking behaviour.

In order to identify and characterize the neuropeptides that are modulated by feeding and are involved in host-seeking, I analysed the antennal lobes, the primary olfactory centre, of *Aedes aegypti*, using semi-quantitative MALDI-TOF mass spectrometry. Functional evidence for the involvement of the identified neuropeptides in the regulation of host-seeking was provided using neuropeptide injections. I demonstrated that short neuropeptide F-2 (sNPF-2) and allatostatin-A-5 (AstA-5) are regulated upon blood feeding and that the injection of a binary mix of sNPF-2 and AstA-5 inhibited host-seeking in non-blood fed mosquitoes, mimicking the effect of a blood meal. I next characterized the sNPF and AstA receptors (sNPFR and AstAR) from *Ae. aegypti* and two other important disease vectors, *Culex quinquefasciatus* and *Anopheles coluzzii* assessing the receptor conservation and function as well as the regulation of the receptors in response to blood feeding. Within the AstA signalling system, I described a dipteran-specific duplication of the AstARs (R1 and R2) in mosquitoes. Functional characterization revealed that the AstAR2s show a higher sensitivity to AstAs compared to AstAR1s in the culicine mosquitoes *Ae.aegypti* and *Cx. quinquefasciatus*. In contrast, both AstARs in *An. coluzzii* showed a similar sensitivity to the AstA ligands, which suggests a divergence in the AstA signalling in mosquitoes. This is in contrast to the sNPFRs in the three species, which showed a high conservation in structure and receptor sensitivity. Blood feeding results in a selective regulation of transcript abundance of the more sensitive *AstAR2* and the *sNPFR* in *Cx. quinquefasciatus*, but not in *Ae. aegypti* or *An. coluzzii*. This is indicative of differences in the regulatory mechanisms for AstA and sNPF in *Cx. quinquefasciatus* compared with the other species.

In this thesis, I provide strong evidence that host-seeking is regulated by complex mechanisms involving at least two neuropeptidergic systems. These findings may shed new light on previous results and should encourage further investigation of other neuropeptide families. The functional characterization of the AstA and sNPF receptors leads to a better understanding of the conservation and regulation of neuropeptide signalling system and provides new targets for future research.

Keywords: olfaction, modulation, neuropeptides, short neuropeptide F, allatostatin-A, host-seeking, sugar feeding, behaviour, G protein coupled receptors

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Dedication

To my family, to my friends, to all the ones who stand by me in times of trouble

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 Peter Christ, Anna Reifenrath, Jörg Kahnt, Frank Hauser, Sharon R. Hill, Joachim Schachtner, Rickard Ignell. Feeding-induced changes in allatostatin-A and short neuropeptide F in the antennal lobes affect odor-mediated host seeking in the yellow fever mosquito, *Aedes aegypti*. submitted

II Peter Christ, Sharon R. Hill, Joachim Schachtner, Frank Hauser, Rickard Ignell. Functional characterization of the dual allatostatin-A receptors in mosquitoes. *Peptides*, accepted with minor revisions

III Peter Christ, Sharon R. Hill, Joachim Schachtner, Frank Hauser, Rickard Ignell. Functional characterization of mosquito sNPF receptors. manuscript

The contribution of Peter Christ to the papers included in this thesis was as follows:

I Assisted in establishing the methodology for the semi-quantitative MALDI-TOF mass spectrometric analysis, carried out all the experiments and data analysis and wrote the manuscript with co-authors.

II Carried out all the experiments and data analysis and wrote the manuscript with co-authors

III Carried out all the experiments and data analysis and wrote the manuscript with co-authors

Abbreviations

AL	antennal lobe
AstA	allatostatin-A
AstAR	allatostatin-A receptor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster oocyte
CNS	central nervous system
CO2	carbon dioxide
DAG	diacylglycerol
DLP	dorsolateral peptidergic neurons
ECL	extracellular loop
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GR	gustatory receptor
GTP	guanosine triphosphate
HP	*Aedes*-Head Peptide
ILP	insulin-like peptide
IP3	inositol triphosphate
IPC	insulin-producing cell
IR	ionotropic receptor
LN	local interneuron
NPF	neuropeptide F
OBP	odorant-binding protein
OR	odorant receptor
OSN	olfactory sensory neuron
PN	projection neuron

1 Introduction

The aim of my PhD work was to explore how host-seeking behaviour of mosquitoes, mainly in *Ae. aegypti*, but also in *Cx. quinquefasciatus* and *An. coluzzii*, is regulated by neuropeptides. More specifically, I asked which neuropeptide families, expressed in the primary olfactory centre, the antennal lobe, of *Ae. aegypti*, are involved in the state dependent regulation of the odour-mediated host-seeking behaviour. Subsequently, I aimed to characterize the cognate receptors of these neuropeptide families in *Ae. aegypti*, *Cx. quinquefasciatus* and *An. coluzzii*, in terms of receptor conservation, function and regulation, following blood feeding.

In this thesis, I provide the background required for the understanding and discussion of my research in the neuropeptidergic regulation of host-seeking in vector mosquitoes. The first part of this thesis provides information about the biology of mosquitoes and their socio-economic implications. Mosquitoes have adapted a life style in which they feed on the blood of other animals for reproduction (Clements, 1992). This is a global problem, since mosquitoes may transmit harmful diseases during blood feeding (WHO, 2015a). In fact, more than half of the human population is at risk to be infected by a mosquito-borne disease, which leads to several million deaths and hundreds of millions of cases of infections every year (WHO, 2015a).

I further outline that which is known about the odour-mediated host-seeking behaviour and its regulation, in mosquitoes. Blood feeding is an integral part of the gonotrophic cycle of female anopheline and culicine mosquitoes, and a successful blood meal leads to a state dependent inhibition of further host-seeking until the gonotrophic cycle resets after oviposition (Klowden, 1990).

Host-seeking relies predominantly on olfactory cues (Clements, 1999; Takken & Knols, 1999; Montell & Zwiebel, 2016), and I provide a short background on the structure and function of the olfactory system, from odour detection at the peripheral level to the integration and modulation in the primary olfactory processing centre, the antennal lobe.

To address the regulation of odour mediated behaviour, I will present that which is known about the neuromodulation of sensory systems, specifically of the olfactory system, and introduce neuropeptides as a large and versatile class of neuromodulatory substances. In this chapter, I also present important neuropeptide families and how they are involved in the nutritional state dependent regulation of feeding behaviour in other insects, focusing on the model organism *D. melanogaster,* where substantial knowledge is available.

The limited information about the neuropeptidergic regulation of host-seeking behaviour in *Ae. aegypti* is discussed. Finally, I will summarize the results of my work, give a general conclusion about the findings and present future perspectives.

2 Biology of Mosquitoes

Mosquitoes (Diptera: Culicidae) encompass approximately 3,500 species that are divided into three subfamilies, the Toxorhynchitinae, Anophelinae and Culicinae (Clements, 1992; Harbach, 2013). While Anopheline and Culicine mosquito species have developed a parasitic lifestyle, in which females have to acquire blood as an essential protein source for egg development and reproduction (haematophagy) (Clements, 1992), mosquitoes from the subfamily Toxorhynchitinae and all male mosquitoes do not feed on blood (Stone, 2013; Lutz *et al.*, 2017). While most haematophagic mosquito species have adapted to feed on non-human hosts (zoophagic), several species have adapted to exclusively feed on humans (anthropophagic) or switch their feeding preference between animal and human hosts (opportunistic) (Takken & Verhulst, 2013). Host preference is mostly genetically determined, but factors like host abundance and the nutritional state of the mosquito can overcome innate host preference (Lyimo & Ferguson, 2009; Takken & Verhulst, 2013).

Whilst most mosquito species are considered to be merely nuisance pests of humans, approximately 100 species are vectors of pathogens, causing a number of perilous human diseases (Becker *et al.*, 2010). This thesis focuses on three mosquito species, *Ae. aegypti*, *Cx. quinquefasciatus* and *An. coluzzii*, with a focus on *Ae. aegypti*. All three species are vectors of important arthropod-borne diseases, which cause substantial loss of life, long-lasting disabilities, and heavy economic burdens on affected countries (WHO, 2015a).

2.1 Investigated mosquito species

2.1.1 Aedes aegypti

Aedes aegypti is a diurnal species, closely associated with humans and their habitation, which exhibits endophagic (indoor feeding) and endophilic (indoor resting) behaviour in its natural environment (Becker *et al.*, 2010). *Aedes aegypti* is highly anthropophilic, with blood meal analysis studies showing that more than 90 % of all blood meals are obtained from humans (Scott *et al.*, 2000; Ponlawat & Harrington, 2005; Scott & Takken, 2012), a preference also observed in laboratory populations (Geier *et al.*, 1996; Bernier *et al.*, 2002).

Field studies show that *Ae. aegypti* can take multiple blood meals within one reproductive cycle (Scott & Takken, 2012). Flight activity, as well as feeding on sugar and blood, peak in the early and late photophase but occur throughout the photophase, albeit at a lower frequency (Jones, 1981; Yee & Foster, 1992). All of these behaviours increase the probability of mosquito-borne disease transmission, contributing to the significance of *Ae. aegypti* as a disease vector.

Aedes aegypti is a vector of a number of viral diseases, including dengue fever, yellow fever, Chikungunya, and Zika (WHO, 2015a). In 2013, an estimated 390 million dengue infections occurred, which manifested clinically in 96 million cases, of which 500,000 were severe cases leading to approximately 20,000 deaths (Bhatt *et al.*, 2013). In the same year, approximately 130,000 cases of yellow fever were reported, causing 78,000 deaths (Garske *et al.*, 2014). The Zika virus received little attention in the medical literature until it recently arrived in Brazil, where between 440,000 and 1,300,000 cases were reported in 2015 (WHO, 2015b). Zika can cause Guillain-Barré syndrome in adults and microcephaly in new-borns whose mothers were infected during pregnancy (Russo *et al.*, 2017). Today, safe and effective vaccines exist only for yellow fever (Barrett, 2017). However, the success of this vaccine has been limited by the lack of sufficient vaccination coverage, as witnessed by recent outbreaks in Angola in 2015 (WHO, 2016a) and in Brazil in 2017 (WHO, 2017).

Due to its significance as a disease vector, this mosquito's host-seeking behaviour, and the regulation of that behaviour, have been studied in some details (Judson, 1967; Klowden & Lea, 1979a; b; Clements, 1999), laying the groundwork for in-depth functional studies, such as those performed in this thesis.

2.1.2 Anopheles coluzzii

Anopheles coluzzii (formerly *Anopheles gambiae* molecular M form) is a species within the *An. gambiae* species complex, which consists of eight morphologically identical species (Coetzee *et al.*, 2013). The M (Mopti) form, together with the Savannah (S) form, were previously considered two molecular forms of *An. gambiae sensu stricto* (Coetzee *et al.*, 2013). This change in nomenclature is slowly being integrated into the research literature. In this thesis, I will use *An. coluzzii* to refer to the former *An. gambiae* molecular M form, albeit the initial reports were published with reference to *An. gambiae.*

In contrast to most other species within the *An. gambiae* complex, *An. coluzzii* is highly anthropophilic (Takken & Knols, 1999; Besansky *et al.*, 2004; Scott & Takken, 2012). This preference for human hosts is also linked to an endophilic and endophagic adaptation (Besansky *et al.*, 2004; Scott & Takken, 2012). *Anopheles coluzzii* is nocturnally active; flight activity and sugar- and blood-feeding are observed almost exclusively during the scotophase (Jones & Gubbins, 1978; Gary & Foster, 2006).

The anthropophilic behaviour of *An. coluzzii* and its high susceptibility for parasitic infection, makes it one of the most important vectors of malaria within the *An. gambiae* species complex (Lyimo & Ferguson, 2009). Malaria is caused by human protozoan *Plasmodium* parasites, predominantly *P. falciparum* and *P. vivax* (Tuteja, 2007), and is one of the greatest disease burdens of tropical and sub-tropical regions (WHO, 2016b). In 2015, 212 million new cases were reported, causing 429 000 deaths (WHO, 2016b). The malaria burden is carried primarily by sub-Saharan African regions, which accounts for 90 % of all cases (WHO, 2016b). Despite a long history of attempts, no effective vaccine has so far been developed (Matuschewski, 2017). Malaria prevention drugs exist, which, however, are expensive and lose efficiency through development of drug resistance of the parasite (WHO, 2016b). The current measure of mosquito control includes insecticide-treated nets and indoor residual spraying, which have proved to be effective in reducing the disease transmission. The progress in disease control, however, is threatened by emerging resistance to insecticides and development of avoidance behaviour by the mosquitoes (WHO, 2016b).

As for *Ae. aegypti*, detailed information is available about the host-seeking behaviour and its regulation in *An. coluzzii*, facilitating comparison between the species (Klowden & Briegel, 1994; Takken *et al.*, 2001).

2.1.3 *Culex quinquefasciatus*

In contrast to *Ae. aegypti* and *An. coluzzii*, *Culex quinquefasciatus* is not strictly anthropophilic, but may feed on a variety of host species depending on geographical and seasonal variation in host choice (Lyimo & Ferguson, 2009; Farajollahi *et al.*, 2011). Host preference in *Cx. quinquefasciatus* differs between populations; in some regions, *Cx. quinquefasciatus* feeds predominantly on humans, while in others, birds are the preferred hosts (Farajollahi *et al.*, 2011; Takken & Verhulst, 2013). Moreover, host preference can change in response to environmental factors, e.g., during bird migrations, making *Cx. quinquefasciatus* an important bridge vector for otherwise primarily avian pathogens (Kilpatrick *et al.*, 2006; Hamer *et al.*, 2008; Farajollahi *et al.*, 2011). *Culex quinquefasciatus* is active primarily at night and also shows a strong activity peak at dusk and dawn (Jones & Gubbins, 1979; Yee & Foster, 1992). Feeding behaviour is mostly endophagic and endophilic, but female mosquitoes have been observed biting humans outdoor as well (Subra, 1981; Becker *et al.*, 2010).

Culex quinquefasciatus is the vector of human lymphatic filariasis, caused by the parasitic nematode *Wuchereria bancrofti*, which in chronic cases leads to elephantiasis (Turell, 2012). Lymphatic filariasis currently threatens almost a billion people living in endemic areas. The disease is not fatal but can lead to profound disfigurement (Turell, 2012). Medical treatment is limited to large-scale preventive chemotherapy to eliminate the disease in affected areas. This mosquito is also a vector of the viral disease West Nile Fever (Turell, 2012). West Nile Fever is caused by an arbovirus with an enzootic cycle between birds and mosquitoes, which can also be transmitted to humans and domestic animals (Kramer *et al.*, 2008). Although approximately 80% of infections are asymptomatic, in one fifth of cases the disease takes a severe debilitating and often encephalitic course (WHO, 2011).

2.2 Odour-mediated behaviours of mosquitoes

The behaviour of mosquitoes and other animals ultimately evolves around two major drives: survival and reproduction. A female mosquito can reproduce several times during its adult life and each time goes through a series of stereotypic behaviours, which collectively constitute the gonotrophic cycle (Klowden, 1990; Klowden & Briegel, 1994). This gonotrophic cycle includes host-seeking, blood feeding and digestion, ovarian development, oviposition site search and the laying of mature eggs (Klowden, 1990; Clements, 1999). All of these behaviours are dependent on sensory systems to detect relevant information from the environment (Montell & Zwiebel, 2016). Of the sensory

systems available, olfaction is considered the most important system in regulating the behaviours of most mosquito species (Clements, 1999; Takken & Knols, 1999; Montell & Zwiebel, 2016). Here, emphasis is placed on odour-mediated feeding behaviours, due to their significance for the understanding of this thesis.

2.3 Odour-mediated feeding behaviours

2.3.1 Sugar seeking

Plant sugars are the main food source for maintaining energy reserves during the adult stage of male mosquitoes and, to some extent, of females (Foster, 1995; Müller & Schlein, 2005). Plants release a complex mixture of volatiles (Nyasembe & Torto, 2014), and a variety of these compounds have been shown to elicit responses in the antennae in *Ae. aegypti* (Jhumur *et al.*, 2007, 2008), *An. coluzzii* (Nyasembe *et al.*, 2012) and *Cx. quinquefasciatus* (Jhumur *et al.*, 2007, 2008). Floral cues are used by the mosquito to identify and discriminate between plant species, and mosquito species often show a clear behavioural preference for specific plants over others (Jhumur *et al.*, 2006; Gouagna *et al.*, 2010; Müller *et al.*, 2010; Otienoburu *et al.*, 2012; Nikbakhtzadeh *et al.*, 2014; von Oppen *et al.*, 2015; Yu *et al.*, 2015). This preference seems to be attributed to a small subset of compounds, as shown in studies using artificial odour blends (Nyasembe *et al.*, 2012; Otienoburu *et al.*, 2012; von Oppen *et al.*, 2015).

2.3.2 Blood seeking

Olfactory cues play a major role in host detection and selection, as well as discrimination between different host species and between individuals of the same host species (Takken & Knols, 1999; Zwiebel & Takken, 2004; Cardé, 2015; Montell & Zwiebel, 2016). All three species are highly attracted to odours, e.g., collected from human skin (Geier & Boeckh, 1999; Takken & Knols, 1999; Pates *et al.*, 2001). Humans, the preferred host for *Ae. aegypti*, *An. coluzzii* and for some populations of *Cx. quinquefasciatus* emit more than 300-400 volatile compounds (Bernier & Kline, 2000). Several of these compounds have been found to be detected by, and to elicit an attractive response in these mosquito species. Examples of these include L-lactic acid, 1-octen-3-ol, ammonia, and carbon dioxide (Geier *et al.*, 1996; Bernier *et al.*, 2002, 2007; Dekker, 2005; Majeed *et al.*, 2017). It is worth mentioning that

most compounds are not active alone, but a blend with the right composition is necessary to elicit a response (Bernier *et al.*, 2007; Majeed *et al.*, 2017).

2.4 Mosquito feeding behaviour within the gonotrophic cycle

Animal behaviour is not static, but must be able to adapt to rapidly changing conditions. This does not only include external, but also internal changes (Bargmann & Marder, 2013; Gadenne *et al.*, 2016). For mosquitoes, the behaviour changes during the gonotrophic cycle in response to factors like age, nutritional estate and the development of ovaries (Clements, 1992, 1999). Most salient are the changes in sugar and blood feeding, which are limited to certain times within the gonotrophic cycle and are dependent on the internal state of the mosquito.

2.4.1 Sugar feeding in the early imago

Nectar from plants is the main sugar source for mosquitoes (Foster, 1995), and *Ae. aegypti*, *Cx. quinquefasciatus* and *An. coluzzii* have been regularly reported feeding on floral and extra-floral nectaries (Müller & Schlein, 2005). Sugar obtained from these resources is an important and readily accessible energy resource and is used to maintain the energy balance of the mosquito, but sugar alone is not sufficient for egg development. Nevertheless, sugar feeding has been shown to increase the survival and fecundity of mosquitoes (Nayar & Sauerman, 1975; Magnarelli, 1978; Straif & Beier, 1996; Manda *et al.*, 2007). Sugar is digested rapidly and can be directly utilized for flight activity (Nayar & Sauerman, 1971; Briegel *et al.*, 2001). In addition, sugar may be transformed by the mosquito into glycogen and triglycerides and stored as energy reserves (van Handel, 1965; Foster, 1995; Naksathit *et al.*, 1999b).

Sugar feeding is observed throughout the gonotrophic cycle, but is of greater importance during the first days of the adult stage (Foster, 1995; Clements, 1999; Foster & Takken, 2004). During this time, the previtellogenic phase of ovarian development takes place, which requires the availability of a sufficient amount of stored nutrients (Clements, 1992). Moreover, for freshly emerged mosquitoes, sugar is the only energy resource, as their blood feeding capacity is not yet developed (Davis, 1984b; Clements, 1999), which, under laboratory conditions, is also reflected by teneral mosquitoes being more attracted to nectar-related odours than to host cues (Foster & Takken, 2004).

For adult *Ae. aegypti* and *An. coluzzii* that are unusually small as a result of limited availability of nutrients during larval development (Takken *et al.*, 1998) an initial sugar meal is necessary to initiate ovarian development (Briegel & Horler, 1993; Takken *et al.*, 1998; Gary & Foster, 2001). However, even large mosquitoes feed on sugar within the first days after adult emergence, which facilitates the subsequent host-seeking behaviour, as observed in *An. coluzzii* (Gary & Foster, 2006) and in some *Aedes* and *Culex* species (Hancock & Foster, 1997; Takken *et al.*, 1998; Briegel *et al.*, 2001; Foster & Takken, 2004; Fernandes & Briegel, 2005).

2.4.2 Blood feeding behaviour in non-gravid females

Blood is a rich source of protein, which is required by haematophagous mosquitoes for ovarian development and egg production. Substantial amounts of nutrients from the first blood meal are sequestered in the ovaries and used for vitellogenesis (Briegel, 1990a; Hancock & Foster, 1993). This is independent of the energy reserves of the mosquito, indicating that the importance of a blood meal primarily lies in the production of eggs (Hancock & Foster, 1993; Naksathit *et al.*, 1999a). In large-sized *Ae. aegypti* and *An. coluzzii* mosquitoes, with access to sugar as an additional energy resource, one blood meal is sufficient to complete a reproductive cycle (Naksathit *et al.*, 1999b; Takken *et al.*, 2001). Nevertheless, blood may also be utilized by the mosquito to fill their energy stores (van Handel, 1965; Clements, 1992; Foster, 1995). Energetically, blood and sugar are interchangeable, but blood digestion is metabolically less efficient than sugar feeding (van Handel, 1965; Clements, 1992; Foster, 1995).

Mosquitoes gradually develop the capacity to host-seek and blood-feed, which in *Ae. aegypti* occurs within 4 days of adult emergence (Davis, 1984a; Clements, 1999). In *An. coluzzii*, blood feeding starts at approximately 40 h post-adult emergence (Takken *et al.*, 1998; Fernandes & Briegel, 2005), and in *Cx. quinquefasciatus* it starts between 28 and 60 h post-emergence (Subra, 1981). After developing the competence to take their first blood meal, the mosquitoes show a stronger preference for host cues compared to plant cues (Foster, 1995; Foster & Takken, 2004).

A complete blood meal leads to long-lasting and extensive changes in the physiology of the mosquitoes, as well as in their feeding and host-seeking behaviour. Nutrients from the blood meal are utilized for the continuation of ovarian and egg development, which requires approximately 3-4 days in *Ae. aegypti*, *An. coluzzii* and *Cx quinquefasciatus* (Subra, 1981; Klowden & Blackmer, 1987; Takken *et al.*, 2001). Several laboratory studies of *Ae. aegypti*

have shown that feeding to completion on blood abolishes further response to host cues until the eggs are deposited (Judson, 1967; Klowden & Lea, 1979b; Takken *et al.*, 2001; Liesch *et al.*, 2013). Host-seeking behaviour can resume, however, if insufficient amounts of blood are consumed – that is, after a partial blood meal (Klowden & Lea, 1979b). The response to host cues in *An. coluzzii* is inhibited for 40 h post-blood meal and then gradually returns until it is completely restored at the time of the onset of oviposition site-selection behaviour (Takken *et al.*, 2001). In undernourished *An. coluzzii*, an initial blood meal is only sufficient to complete previtellogenic ovarian development, in which case, a second blood meal is required to complete a full gonotrophic cycle (Feinsod & Spielman, 1980; Briegel, 1990b; Takken *et al.*, 1998).

Not only blood feeding, but also sugar feeding is inhibited following a complete blood meal (Gary & Foster, 2006). During early ovarian development, *An. coluzzii* rarely feeds on sugar (Vargo & Foster, 1982, 1984; Gary & Foster, 2006), and in *Ae. aegypti*, attraction to floral compounds and sugar-feeding during ovarian development has seldom been observed in wild animals (Vargo & Foster, 1982, 1984). In contrast, in sugar-starved *Ae. aegypti* under laboratory conditions, host-seeking is restored already at the semi-gravid state (48 h post-blood meal), which indicates that the mosquito needs to replenish its energy resources at this stage of the gonotrophic cycle (Klowden, 1986).

In summary, feeding behaviours in mosquitoes are inhibited after a successful blood meal, and returns over time in a species-specific manner. While in *Ae. aegypti* only sugar feeding returns, *An. coluzzii* has been observed host-seeking already in pre-gravid conditions.

2.4.3 Blood or sugar feeding in gravid females

Generally, gravid mosquitoes benefit from taking another meal of sugar or blood before oviposition behaviour is initiated (Klowden, 1986; Takken *et al.*, 2001; Styer *et al.*, 2007). The resource chosen appears to be dependent on the availability of food sources in the environment. In *Ae. aegypti*, host-seeking behaviour of gravid mosquitoes remains inhibited until eggs are laid (Judson, 1967; Klowden & Lea, 1979b; Takken *et al.*, 2001; Liesch *et al.*, 2013), but only if sugar as a food source is provided (Klowden, 1986). In contrast, in the field, several studies on *Aedes* species indicate that sugar feeding is considerably less frequent in the presence of humans (Edman *et al.*, 1992; van Handel *et al.*, 1994; Martinez-Ibarra *et al.*, 1997; Spencer *et al.*, 2005), suggesting that a second blood meal is commonly taken to replenish energy

resources (Scott & Takken, 2012). In the absence of humans, however, the sugar-feeding rate increases to 74 % (van Handel *et al.*, 1994).

Similar observations have been made in *An. coluzzii* (Klowden & Briegel, 1994; Takken *et al.*, 2001; Gary & Foster, 2006). This indicates that experiments under laboratory conditions should to be conducted while carefully controlling the feeding regime

2.4.4 Blood and sugar feeding after oviposition

After egg-laying, the gonotrophic cycle resets, and mosquitoes commonly take a sugar meal to replenish their energy resources (Gary & Foster, 2006). In *An. coluzzii*, the response to host cues is restored before oviposition (Takken *et al.*, 2001). In *Ae. aegypti*, on the other hand, the acceptance of a blood meal returns gradually, with only 10 % of mosquitoes taking a blood meal directly after oviposition, increasing to 100 % at ca. 24-46 h after oviposition (Chadee, 2012). In addition, *Ae. aegypti* and *An. coluzzii* regain behavioural responsiveness towards host cues during this time (Judson, 1967; Klowden & Blackmer, 1987; Takken *et al.*, 2001).

2.5 Regulation of host-seeking behaviour

As discussed above, a successful, complete blood meal leads to significant changes in the physiology and behaviour of mosquitoes, including a transient inhibition of the odour-mediated host-seeking behaviour. The mechanism underlying this behavioural inhibition has been studied in some detail in *Ae. aegypti*, in which it has been divided into two phases: an immediate inhibition that starts directly after a blood meal and lasts for up to 24 hours post-blood feeding, followed by a delayed inhibition that continues until the onset of pre-oviposition behaviour, approximately 72 hours post-blood meal (Clements, 1999).

2.5.1 The immediate phase of host-seeking inhibition

A complete blood meal results in a large distension of the abdomen of the mosquito. In a series of studies, Klowden and Lea showed that this distension triggers the immediate inhibition of host-seeking behaviour in *Ae. aegypti*. By comparing different blood-meal sizes, they were able to show that only large volumes of blood (2.5 - 4 µl) trigger immediate host-seeking inhibition (Klowden & Lea, 1978). A large blood meal can be simulated by inflating the abdomen with saline or air (Klowden & Lea, 1979a). Preventing movement of

different parts of the abdomen using melted wax indicates that distension of the anterior region is responsible for the observed inhibition (Klowden & Lea, 1979a). Based on these results, the authors reasoned that the immediate inhibition is triggered by stretch-sensitive neurons in the anterior half of the abdomen (Klowden & Lea, 1979a). In addition, nerve cord transection at the 2nd abdominal ganglion prevented the immediate host-seeking inhibition, suggesting that the stretch receptors are situated in the thorax above the transection (Klowden & Lea, 1979a; Klowden, 1990).Alternatively, the abdominal distension could trigger the release of a neuromodulatory substance via an unknown pathway (Klowden & Lea, 1978, 1979a; Klowden, 1990). Although it has been studied in detail only in *Ae. aegypti*, (Klowden & Lea, 1978, 1979a; Clements, 1999), immediate inhibition of host-seeking behaviour by abdominal distension has also been suggested for other *Aedes* and *Anopheles* species (Klowden & Briegel, 1994).

2.5.2 The delayed phase of host-seeking inhibition

Two observations suggest that the delayed inhibition of host-seeking behaviour is regulated independently of the immediate phase of inhibition (Klowden & Lea, 1979b). First, in fully gorged mosquitoes, inhibition of host-seeking persists after abdominal distension ends (Klowden & Lea, 1979b; Klowden, 1990). Second, female *Ae. aegypti* develop unresponsiveness to host cues only after 24 h, when digesting a small volume of blood, under the condition that egg development is triggered (Klowden & Lea, 1979b; Klowden, 1990). The observation that lymph-transfusion from a blood-fed to a non-blood-fed animal mosquito leads to inhibition of host-seeking when tested 2 h post-transfusion, suggests that a haemolymph-borne factor is responsible for regulating the delayed inhibition (Klowden & Lea, 1979b).

These authors also showed that the ovaries are important for the delayed host-seeking inhibition, as ovariectomy in *Ae. aegypti* before a blood meal prevents the delayed inhibition. In addition, transplantation of fat bodies from females 24 h post-blood feeding into non-blood-fed females leads to a blood-fed phenotype 24 h later, suggesting that the fat body is the source of the haemolymph-borne factor (Klowden *et al.*, 1987; Klowden, 1990). Interestingly, fat bodies from blood-fed *Cx. quinquefasciatus* also lead to a reduction of host-seeking behaviour in *Ae. aegypti* (Klowden *et al.*, 1987).

So far there is only limited information about the identity of this humoral factor (Clements, 1999). Brown *et. al*. (1994) found the neuropeptide *Aedes*-Head-peptide I (HP) to be a candidate humoral factor, showing that the titre of the peptide increased after a successful blood meal, and demonstrating that

peptide injection led to inhibition of host-seeking behaviour. However, later studies were unable to detect this neuropeptide in female *Ae. aegypti*; see chapter *5.6* for further discussion on this. Humoral inhibition of host-seeking behaviour is terminated with oviposition. This termination seems to be triggered by a nervous pathway, originating from the ovaries (Klowden, 1981).

3 The olfactory pathway

As stated above, mosquitoes rely heavily on their sense of smell to locate and select food sources (Clements, 1999; Takken & Knols, 1999; Zwiebel & Takken, 2004; Montell & Zwiebel, 2016). Information in the form of a bouquet of odour molecules has to be detected, processed, and integrated with the animal's physiological state and experience before it is ultimately translated into an appropriate behavioural output (Martin *et al.*, 2011; Wicher, 2015; Gadenne *et al.*, 2016). The olfactory organs of mosquitoes are the antennae, maxillary palps, and labella (Keil, 1999). These organs are covered with various forms of cuticular hair-like structures called sensilla that house the olfactory sensory neurons (OSNs). From their cell bodies located at the base of each sensillum, these neurons extend dendrites responsible for the detection of odour molecules into the lymph within the cuticular bristle. Their axons project to the primary olfactory processing centre, the antennal lobe (AL), where they synapse onto the dendrites of projection neurons (PNs). This neuropil is organised into spherical structures called glomeruli, which are interconnected via local interneurons (LNs). Initial sensory integration and processing occurs in the AL, mediated by the LNs. The PNs relay the information to higher brain centres where it is integrated with other sensory inputs and the experience of the animal and ultimately translated into a behavioural output (Martin *et al.*, 2011).

3.1 The peripheral olfactory system

The first step in insect olfaction is odour detection, which takes place in the olfactory sensilla, which make up 90 % of the sensilla on the antennae (McIver, 1978, 1982). Olfactory sensilla are divided into two distinct types – single-walled and double-walled – but may be further subdivided into different morphological classes (Keil, 1999). In mosquitoes, there are three

morphological classes of olfactory sensilla, the single-walled *sensilla trichodea* and the double-walled grooved pegs and *sensilla coeloconica* (McIver, 1982; Pitts *et al.*, 2004; Hill *et al.*, 2009). This last class, however, is only present in anophelines (McIver, 1982). All olfactory sensilla have numerous pores (*sensilla trichodea*) or spokes (*sensilla coeloconica* and grooved pegs), through which environmental odours reach the interior of the sensillum. The interior of the sensillum contains the sensillar lymph and the dendrites of one or more bipolar OSNs, expressing olfactory receptors (Keil, 1982, 1999; Steinbrecht, 1997; Stengl *et al.*, 1999). The axons of the OSNs are bundled in the antennal nerve and project into the AL (Anton & Homberg, 1999; Schachtner *et al.*, 2005). In addition to the OSN cell bodies, the base of each olfactory sensillum houses three accessory cells, the thecogen, trichogen and tormogen cells. These cells are involved in establishing an adequate ionic environment within the sensillum and expressing auxiliary proteins, such as the odorant-binding proteins (OBPs), which play a role in the signal transduction (Keil, 1999; Stengl *et al.*, 1999; Leal, 2013).

3.1.1 Odour detection in the periphery

The odour detection in the periphery must be rapid and accurate, and is realised by the auxiliary proteins in the sensillum environment and the properties of the OSNs. The passage of odorants through the sensillar lymph is an initial filtering step and is believed to be mediated by OBPs that form a complex with the odorants and shuttle these generally hydrophobic molecules through the aqueous sensillar lymph (Leal, 2013). Recent studies, however, indicate that OBPs are not always essential for odour detection and can have other functions in the sensillum, such as early gain control (Leal, 2013; Larter *et al.*, 2016). In support of this, many studies have shown that specificity of the odorant detection generally is mediated by olfactory receptors that bind the odorants (Dahanukar *et al.*, 2005; Hallem & Carlson, 2006; Carey *et al.*, 2010).

Three major classes of olfactory receptors have been described to date (Guidobaldi *et al.*, 2014; Wicher, 2015), and will be discussed here in more detail, due to their importance in the olfactory pathway. The olfactory receptors include the large class of odorant receptors (ORs), their closely related homologues, the gustatory receptors (GRs), and the ionotropic receptors (IRs) (Kaupp, 2010; Guidobaldi *et al.*, 2014; Wicher, 2015).

3.1.2 Odorant receptors (ORs)

The insect ORs contain seven transmembrane domains, but are distinct from other seven-transmembrane-domain receptor classes such as the G-protein coupled receptors (GPCRs) or the vertebrate ORs (Nakagawa & Vosshall, 2009; Silbering & Benton, 2010). Characteristic for insect ORs is their inverted topology relative to other GPCRs, with the N-terminus located inside and the C-terminus outside of the cell (Benton, 2006; Lundin *et al.*, 2007). Unique ORs are co-expressed with a ubiquitous co-receptor, named "Orco" (Larsson *et al.*, 2004; Pitts *et al.*, 2004; Vosshall & Hansson, 2011), which is required for the localization of the ORs in the dendritic membrane and their subsequent function (Larsson *et al.*, 2004; Benton, 2006; Wicher *et al.*, 2008; Mukunda *et al.*, 2014). The OR and Orco together form a heterodimeric complex, which can act as an ionotropic channel and/or a metabotropic receptor (acting through a second messenger cascade) (Sato *et al.*, 2008; Wicher *et al.*, 2008; Deng *et al.*, 2011; Wicher, 2013). A concrete model for OR-Orco function is, however, still debated (Stengl & Funk, 2013; Wicher, 2013, 2015).

According to the convention, OSNs are generally said to express only one type of tuning OR together with Orco (Galizia & Sachse, 2010; Sachse & Krieger, 2011). However, exceptions to this rule have been reported. One example of this is described in *An. coluzzii*, where co-expression of several OR genes has been observed (Karner *et al.*, 2015).

Insect tuning ORs are highly diverse between species in number, and sequence diversity (Suh *et al.*, 2014). In *Ae. aegypti* there are 129 annotated ORs, with 100-110 ORs demonstrated to be expressed in the adult female antennae (Bohbot *et al.*, 2007; Bohbot & Pitts, 2015; Matthews *et al.*, 2016), while in *Cx. quinquefasciatus* 180 ORs have been annotated, of which 96 are expressed in adult female antennae (Arensburger *et al.*, 2010; Taparia *et al.*, 2017). In contrast, in *An. coluzzii* only 58 genes are expressed in the antennae, out of a total number of 79 genes (Hill *et al.*, 2002; Rinker *et al.*, 2013). The different numbers of ORs expressed in mosquito species is a result of several species-specific OR expansions (Bohbot *et al.*, 2007; Arensburger *et al.*, 2010). Large expansion of the OR gene repertoire appears to be characteristic of culicine mosquitoes, potentially reflecting culicine olfactory behavioural diversity, seen e.g. as a reflection of the opportunistic feeding behaviour of *Cx. quinquefasciatus* (Arensburger *et al.*, 2010).

While early studies on ORs indicated that many ORs responded to a broad range of odours, recent studies indicate that the majority of the ORs are narrowly tuned, with a high sensitivity to a single compound or a narrow group of compounds (Suh *et al.*, 2014; Andersson *et al.*, 2015; Bohbot & Pitts, 2015). Several studies have also shown that the ORs in specific insect species are

most often found to be tuned to compounds that are ecologically relevant for the species (Hallem & Carlson, 2006; Carey *et al.*, 2010; de Fouchier *et al.*, 2017). For example, already in pioneering OR deorphanization studies, it was shown that the odour space covered by narrowly tuned receptors in *D. melanogaster* is more focused on esters when compared to that of *An. coluzzii*, which allocated greater relative coverage to aromatics (Hallem & Carlson, 2006; Carey *et al.*, 2010). This observation was hypothesised to be an adaptation of these species to detect and discriminate between relevant odours, used for example for food seeking. Esters are common compounds of fruits, while several aromatics are compounds of human sweat (Carey *et al.*, 2010). Recent studies also suggest a model in which the repertoire of narrowly tuned ORs in each species have evolved to be highly specific for odorants of ecological importance for this insect (Andersson *et al.*, 2015). For example, a recent investigation in the moth *Spodoptera littoralis* indicated that the OR repertoire of this herbivore is generally tuned towards odour classes found in plants, and that several specialist ORs are highly sensitive to specific plant-related volatiles (de Fouchier *et al.*, 2017). In addition, several studies in mosquitoes have indicated the existence of narrowly tuned ORs for compounds that mark oviposition sites or host animals (Guidobaldi *et al.*, 2014). The rapid evolution of narrowly tuned ORs has been demonstrated in a study comparing an anthropophilic urban strain of *Ae. aegypti* with a zoophilic forest strain of this species (McBride *et al.*, 2014). The authors showed differential expression and sensitivity of the narrowly tuned OR4 in the two sub-species. This OR responds to the compound sulcatone, which is enriched in the human headspace compared to a variety of animals (McBride *et al.*, 2014). The tuning of the ORs to behaviourally relevant compounds, together with their rapid evolution, show two key features of the olfactory system, which needs to be very specific to fitness-related cues, but on the other hand adaptable to changes of environmental conditions.

3.1.3 Gustatory receptors (GRs)

The GRs have a common lineage to the ORs, but represent a more basal group of insect chemoreceptors, which are generally devoted to the sense of taste, meaning contact chemoreception (Hill *et al.*, 2002). Nevertheless, in insects, the detection of the important mosquito host cue, carbon dioxide (CO_2), is realized by highly conserved members of the GR family, which reflects the importance of this ubiquitous sensory cue that plays a role in multiple insect behaviours (Guerenstein & Hildebrand, 2008). In mosquitoes, the CO_2-sensitive GRs are expressed in the non-OR-expressing OSN found in capitate

peg sensilla on the maxillary palps (Lu *et al.*, 2007; Robertson & Kent, 2009; Erdelyan *et al.*, 2012). These CO_2-sensitive OSNs house a trio of GRs (*An. coluzzii*: GR22, GR23 and GR24 and *Ae. aegypti* GR1, GR2 and GR3; Lu *et al.*, 2007; Erdelyan *et al.*, 2012). It was shown for *Ae. aegypti* and *D. melanogaster* that only two GRs (*Ae. aegypti* GR1 and GR3 and their orthologues) are necessary for CO_2 detection (Jones *et al.*, 2007; Erdelyan *et al.*, 2012).

3.1.4 Ionotropic receptors (IRs)

The IRs are chemoreceptors derived from a different superfamily, the ionotropic glutamate receptors, which are considered more ancestral compared to the ORs, and only a subset of the IRs, the so called antennal IRs, are involved in odour detection (Rytz *et al.*, 2013). In early anatomical and functional studies in *D. melanogaster,* OSNs in *sensilla coeloconica* were found to be devoid of ORs or GRs (Yao *et al.*, 2005; Vosshall & Stocker, 2007). In 2009, Benton *et al.* suggested the IRs as the missing olfactory receptors expressed by those OSNs. Subsequently, Pitts *et al.* (2017) found *An. coluzzii* IRs to be expressed in OSNs of grooved peg sensilla. These sensilla are morphologically analogous to the *sensilla coeloconica* of *D. melanogaster* (Benton *et al.*, 2009).

Ionotropic receptors exhibit an overall topology resembling that of ionotropic glutamate receptors (Liu *et al.*, 2010; Rytz *et al.*, 2013). The amino-terminal and extracellular domains are the largest parts of the olfactory IRs and contain a distinct ligand-binding domain, while the membrane bound part of the receptor forms an ion channel domain (Rytz *et al.*, 2013). Unlike the ORs, which dimerize with the single co-receptor Orco, IRs share three highly conserved co-receptors. Functional studies have shown that the olfactory IRs may form a complex with one or more of these, functioning together as a ligand-gated ion channel (Croset *et al.*, 2010; Rytz *et al.*, 2013; Pitts *et al.*, 2017).

The number of total IRs expressed in mosquitoes, differs between species with 95 in *Ae. aegypti*, 69 in *Cx. quinquefasciatus*, and 46 in *An. coluzzii* (Croset *et al.*, 2010; Matthews *et al.*, 2016; Pitts *et al.*, 2017; Taparia *et al.*, 2017). Only a subset of these are however expressed on the antennae (antennal IRs).The number of antennal IRs in the culicines (29-30), is almost one third higher than in the anophelines (23) (Croset *et al.*, 2010; Matthews *et al.*, 2016; Pitts *et al.*, 2017; Taparia *et al.*, 2017). In contrast to the family of ORs, antennal IRs are conserved in insects, in both sequence and expression patterns (Croset *et al.*, 2010). Several antennal IRs described in *D. melanogaster*

respond to amine and carboxylic-acid odorants. A recent study in *An. coluzzii* deorphanized several IR complexes homologous to IRs previously described in *D. melanogaster*, finding responses to a narrow range of amines and carboxylic acids, supporting a functional conservation of those IR complexes in detecting these odour groups (Ai *et al.*, 2010; Abuin *et al.*, 2011; Silbering *et al.*, 2011; Hussain *et al.*, 2016; Pitts *et al.*, 2017). Interestingly, several of the identified compounds are found in human sweat (Bernier & Kline, 2000), suggesting that the *An. coluzzii* IRs are tuned towards these behaviourally relevant compounds (Pitts *et al.*, 2017).

3.2 The primary olfactory processing centre, the antennal lobe (AL)

The AL is the primary relay centre for olfactory information, where integration and processing occurs. The AL is innervated by the OSNs, which project into distinct spherical subunits of the AL called glomeruli. Detailed molecular and anatomical mapping of OSNs in *D. melanogaster* show that all OSNs expressing the same olfactory receptor converge into the same glomerulus (Vosshall *et al.*, 2000; Couto *et al.*, 2005). In other insects, including mosquitoes, this chemotopic organisation of olfactory information has been shown through tracing of functional distinct classes of OSNs into single glomeruli (Ghaninia *et al.*, 2007; Ignell *et al.*, 2010). Each glomerulus receives input from only one OSN type, typically representing one olfactory receptor, according to the one-OSN, one-receptor, one-glomerulus rule (Couto *et al.*, 2005). The sizes, numbers, and positions of glomeruli, however, differ greatly between insect species, as revealed by three-dimensional reconstructions (Huetteroth & Schachtner, 2005; Ignell *et al.*, 2005; Schachtner *et al.*, 2005; Ghaninia *et al.*, 2007; Nishikawa *et al.*, 2008; Dreyer *et al.*, 2010). For instance, 50 glomeruli are found in female and 49 in male *Ae. aegypti* (Ignell et al., 2005), whereas 60 and 61 glomeruli have been found in male and female ALs in *An. coluzzii*, respectively (Ghaninia *et al.*, 2007). Within each glomerulus, the axons of the OSNs make synaptic connections with other neurons in the AL, the LNs and PNs, which are responsible for the processing of the incoming olfactory information.

The chemotopic organisation suggests a functional representation of odorants at the level of the AL (Hansson & Christensen, 1999; Vosshall *et al.*, 2000; Couto *et al.*, 2005). If only narrowly tuned OSNs would innervate each glomerulus, the chemotopic map would be a simple, unequivocal functional map. This is the case for several ecologically important odours such as pheromones (Kurtovic *et al.*, 2007; Ruta *et al.*, 2010; Dweck *et al.*, 2015),

important aversive odours (Stensmyr *et al.*, 2012; Ebrahim *et al.*, 2015), and CO_2 (Jones *et al.*, 2007; Kwon *et al.*, 2007) which appear to have dedicated pathways. However, most OSNs appear to be more broadly tuned, with individual compounds evoking activation in several OSNs in a concentration-dependent manner (Todd & Baker, 1999). As a result, more than one glomerulus can respond to the same odorant, with the pattern of activation being dependent on odorant concentration.

While the integration of olfactory information in the AL is realised by the wiring of the OSNs, the processing is performed by LNs and PNs, which receive synaptic input from OSNs within the glomeruli (Martin *et al.*, 2011). Local interneurons arborize in most if not all AL glomeruli and form synaptic output on OSNs or other LNs, and are mainly responsible for the olfactory information processing in the AL (Sachse & Krieger, 2011). Olfactory information processed in the glomeruli converges on uniglomerular or multi-glomerular PNs, which transmit the pre-processed olfactory information towards higher brain centres (Anton & Homberg, 1999; Martin *et al.*, 2011). In addition, the AL is innervated by extrinsic neurons from other brain areas, which mostly express neuromodulatory substances (Anton & Homberg, 1999).

3.2.1 Projection neurons (PNs)

Projection neurons make synaptic contact with the OSNs and connect to higher brain centres (Galizia & Rössler, 2010; Kaupp, 2010). Two types of PNs are observed, receiving input from either one glomerulus (uniglomerular and mostly excitatory) or less commonly, several glomeruli (multiglomerular and mostly inhibitory) (Galizia & Rössler, 2010; Martin *et al.*, 2011). Within a single glomerulus, a large number of OSNs converge onto a small number of PNs (Grabe *et al.*, 2016). This convergence is an important step in the processing of olfactory information and has been shown to decrease noise and/or strengthen weak signals from OSNs (Galizia, 2014).

3.2.2 Local interneurons (LNs)

Local interneurons are morphologically diverse, with cell bodies located in a lateral, and in some insects also a ventral, cluster at the periphery of the AL (Ignell *et al.*, 2005; Schachtner *et al.*, 2005). Most LNs arborize in many, if not all, glomeruli with variable branching within specific glomeruli (pan-glomerular LNs) (Chou *et al.*, 2010; Reisenman *et al.*, 2011). In addition, a subpopulation of LNs is generally also found to arborize in a small number of specific glomeruli (oligoglomerular LNs) (Chou *et al.*, 2010; Reisenman *et al.*,

2011). So far, in mosquitoes only LNs with homogenous arborisations in most, if not all, AL glomeruli have been described (Ignell *et al.*, 2005).

In insects, most LNs express γ-aminobutyric acid (GABA), although glutaminergic LNs also exist, making interglomerular interaction inhibitory (Homberg & Müller, 1999; Liu & Wilson, 2013). Note that in *D. melanogaster*, but so far not in any other insects, excitatory cholinergic LNs have also been found (Olsen et al., 2007; Shang et al., 2007). In addition, LNs have also been shown to co-express neuromodulatory neuropeptides along with the classical transmitters (Siju *et al.*, 2014; Lizbinski *et al.*, 2017). Typically, these neuromodulators are recruited during high neuronal activation and act on metabotropic receptors (Nusbaum *et al.*, 2017). A core function of LNs in the AL is the processing of the olfactory information through the lateral connection of glomeruli. This includes presynaptic gain control, global normalization, and control of response range (Martin *et al.*, 2011; Wilson, 2013; Galizia, 2014).

An additional role of the LNs is the integration of state-dependent information. Local interneurons have been described to express the receptors for several neuromodulatory substances, including biogenic amines and neuropeptides, which allows for comprehensive neuromodulation of the AL network (Dacks *et al.*, 2013; Rein *et al.*, 2013; Nusbaum *et al.*, 2017). For example, LNs can be recruited to modify signals according to the nutritional state of the animals and modify food-related odour responses in the state of satiety, while leaving other signals unaffected (Ignell *et al.*, 2009; Root *et al.*, 2011; Ko *et al.*, 2015).

3.2.3 Neuromodulatory extrinsic neurons

The AL is innervated by various extrinsic neurons from other brain areas with cell bodies generally outside of the AL, which can interact with the network of processes within the AL (Anton & Homberg, 1999; Schachtner *et al.*, 2005). Extrinsic neurons can vary greatly in morphology and location, and are often found to express neuromodulatory substances, such as biogenic amines (Homberg & Müller, 1999; Siju *et al.*, 2008; Rein *et al.*, 2013) and neuropeptides (Nässel, 2000; Siju *et al.*, 2014). A common characteristic of the extrinsic neurons are wide-reaching arborisations outside the AL (Anton & Homberg, 1999).

Immunohistochemical studies in *Ae. aegypti* have revealed a number of neuromodulatory extrinsic neurons with arborisations within the AL (Siju *et al.*, 2008, 2014). Siju *et al.* (2008) describes a serotonin-immunoreactive neuron, which has arborisations in higher brain centres and most if not all glomeruli in the AL. In addition, several extrinsic neurons have been found to

express neuropeptides (Siju *et al.*, 2014). An example of this is a group of four conserved neurons with cell bodies in the pars intercerebralis expressing the neuropeptide SIFamide (SIFa). These cells innervate the entire brain, including the AL, in a dense meshwork (Verleyen *et al.*, 2004; Heuer *et al.*, 2012; Siju *et al.*, 2014). The neuroanatomy of extrinsic neurons suggests that they might have functions as feed-back neurons from other brain areas to the AL or in the broad modulation of several brain areas, for example in the context of state-dependent modulation (Anton & Homberg, 1999; Martin *et al.*, 2011; Sengupta, 2013).

4 Neuromodulation

Modulation and plasticity of neuronal circuits is a key feature of animals, used to adapt their behaviour to a steadily changing environment (Gadenne *et al.*, 2016). Changes in behaviour can be triggered by external factors, but also in response to the internal state of the animal, e.g. in response to satiety or a change in mating status (Gadenne *et al.*, 2016; Kim *et al.*, 2017). Several examples of state-depended switches in olfactory-guided behaviours have been observed in insects. One example is the mosquito's loss of responsiveness to host-related odours following a blood meal, introduced in the previous chapters of this thesis. Another example is found in the African cotton leaf worm, *S. littoralis*, in which the response to feeding-related flower odours in females is downregulated upon mating, while the response to oviposition-site-related green-leaf odours is upregulated (Saveer *et al.*, 2012). A third and well-investigated example is the response to food-related odours depending on the nutritional state observed in several insects, but in its complexity studied primarily in *D. melanogaster* (Itskov & Ribeiro, 2013; Schoofs *et al.*, 2017).

How is a change of the internal state translated into a change in the behavioural output? These switches in behaviour can be achieved by neuromodulatory substances affecting the neuronal response of sensory systems, e.g. within the olfactory pathway (Wang, 2012; Su & Wang, 2014). For example, the nutritional state of an animal is measured by internal sensors, which may induce the release of, for example, broadly-released biogenic amines or neuropeptides acting as neurohormones (Itskov & Ribeiro, 2013; Kim *et al.*, 2017). An example of a neurohormone is the insulin-like peptide (ILP), which will be discussed in a later chapter in more detail (*5.4.5*). Neurohormones can directly affect neuronal activity, but more commonly, recruit other neuromodulators in local networks that act alongside the classical fast synaptic transmission (Bucher & Marder, 2013; Kim *et al.*, 2017). Typically, a high number of neuromodulatory substances can be found within these local networks (Lizbinski *et al.*, 2017). To give an example, in the AL of

Ae. aegypti and *D. melanogaster*, 10 or 7 different neuropeptide families are found, allowing for a broad modulation of the system (Carlsson *et al.*, 2010; Siju *et al.*, 2014). Neuromodulators can be released in a locally confined manner, e.g. by the LNs of the AL (Nässel, 2009). Here, the neuromodulator is released close to the synaptic cleft and acts either presynaptically or postsynaptically (or both), depending on the expression of the cognate receptor (Nässel, 2009). The neuromodulators, however, are not necessarily limited to a single synapse, but may reach receptors at neighbouring synapses (Nässel, 2009). Additionally, neuromodulators can be released in a paracrine fashion, sometimes called volume transmission, where the neuromodulator is released along the axon or from diffusely arborizing terminations (Nässel, 2009). In some cases the neuromodulator release is not even tied to the local network, but global levels of neuromodulators in the lymph may be detected by the local receptors (Nässel, 2009; Kim *et al.*, 2017). An example for this is ILP, mentioned previously (Nässel & Broeck, 2016). In summary, modulation of local networks is sophisticated and very complex and we only now begin to understand their precise regulation.

5 Neuropeptides

Neuropeptides are diverse in structure and distribution, well conserved during evolution, and involved in a multitude of physiological processes and in the regulation of behaviour (Nässel, 2002; Nässel & Wegener, 2011; Grimmelikhuijzen & Hauser, 2012; Schoofs *et al.*, 2017). Neuropeptides are produced by neurons or neurosecretory cells, and can therefore be specified as neuropeptides or neurohormones (Nässel, 2009). Some, but not all, neuropeptides can be expressed by multiple cell types in the nervous system or the endocrine system (Nässel & Winther, 2010; Wegener & Veenstra, 2015). Neuropeptides are encoded in the genome as larger precursor proteins, referred to as prepropeptides (Fricker, 2012). Prepropeptides are, upon translation, cut at specific cleavage sites and modified into mature neuropeptides (Fricker, 2012). In many cases, several neuropeptides are cleaved from one precursor (neuropeptide isoforms), and in these cases are classified as belonging to the same neuropeptide family (Nässel & Winther, 2010; Coast & Schooley, 2011). Neuropeptides belonging to one family are usually structurally related and possess a conserved motif at the C-terminus, which plays a role in the interaction between the neuropeptide and its corresponding receptor, generally of the GPCR family (Nässel & Winther, 2010). In invertebrates there is typically one receptor for each neuropeptide family (Nässel & Winther, 2010). Recent research, specifically in *D. melanogaster*, has allowed for an increased understanding of neuropeptide signalling in insects.

5.1 Origin and evolution

Neuropeptides are an ancient signalling system, which probably evolved alongside the origin of the nervous system (Grimmelikhuijzen & Hauser, 2012), or even before this (Fairclough *et al.*, 2013). The first animals considered to have a nervous system are from the phylum of *Cnidaria*, which

evolved before the split of Protostomia and Deuterostomia. Interestingly, cnidarians show a rich number of neuropeptides, but are lacking classical (fast-acting) transmitter systems, like acetylcholine and GABA (Grimmelikhuijzen *et al.*, 2002). Many of the ancestral neuropeptide families, however, have changed after years of receptor/peptide coevolution and barely resemble the ancient structure (Hansen *et al.*, 2010). Nonetheless, some neuropeptide families have retained their structure and binding properties, so that in a few cases, an insect neuropeptide receptor can still be activated by, e.g., a mammalian neuropeptide (Birse *et al.*, 2006).

Several studies have striven to identify the ancestors of "modern" neuropeptide families, by analysing neuropeptides in evolutionarily primitive animals (Jekely, 2013; Mirabeau & Joly, 2013). The findings from those studies indicate that many neuropeptide systems originated from their core paralogues, i.e., are derived from the same ancestral gene, but have undergone structural changes during their respective evolution (Jekely, 2013). For example, neuropeptides of many families have a similar C-terminal recognition site bearing an RFamide motif (i.e., an amidated C-terminal arginine-phenylalanine sequence) (Elphick & Mirabeau, 2014). One hypothesis for the occurrence of several structurally related RFamide-signalling systems is that the common ancestor possessed a signalling system ending with RFamide, which duplicated during the course of evolution and diversified. The RFamide motif has in this case been either retained, modified or lost in different animal phyla (Elphick & Mirabeau, 2014) This marks an important aspect of neuropeptide evolution, which is that neuropeptide signalling systems can duplicate, creating new neuropeptide families, or can disappear completely in some orders (Hauser *et al.*, 2008; Hauser & Grimmelikhuijzen, 2014).

Analysis of the evolutionary old insect subclass Pterygota shows that the set of neuropeptide families remains fairly conserved in the class of insects (Derst *et al.*, 2016). Nevertheless, modification events, including modification of sequences, gene duplications, changes of isoforms within neuropeptide precursors, and even the complete loss of a family can be found as lineage-specific events (Derst *et al.*, 2016). One common conserved feature of the neuropeptide signalling systems is their role in regulating sensory input in a state-dependent manner (Bargmann, 2012; Taghert & Nitabach, 2012). Several other conserved functions in insects, are in part shared with the ancestral organisms, for example, muscle control (McFarlane *et al.*, 1987) and regulation of food intake (Dockray, 2004).

5.2 Processing

In contrast to classical neurotransmitters, which are generally enzymatically synthesised directly in the synapse, neuropeptides are generated via standard ribosomal translation in the cell body (Fricker, 2012). Neuropeptide genes are translated into large peptides, called prepropeptides, which subsequently are processed into one or several functional neuropeptides (Fricker, 2012). Prepropeptides contain a secretory signal sequence and recognition sites for enzymatic cleavage separating the neuropeptide moieties. After translation, the prepropeptide is guided into the lumen of the rough endoplasmic reticulum, where the signal peptide is removed (as in the classical secretory pathway) (Fricker, 2012). As a next step, the precursor is packed into secretory granulates (large dense core vesicles) and processed into the mature neuropeptides, in a cascade involving several enzymatic steps. One important step is the enzymatic cleavage of the mature neuropeptides at a recognition site of specific basic amino acids (Veenstra, 2000). In invertebrates, a majority of these recognition sites contains a dibasic sequence of lysine (K) and/or arginine (R). The site KR is most common in insects, although in a few cases RR sites are cleaved as well (Veenstra, 2000). In addition, several precursors contain so called monobasic cleavage sites of R. Usually a monobasic site contains another R upstream after an even number of non-basic amino acids (e.g. RXXR or RXXXXR) (Veenstra, 2000). In this case, the cut is usually introduced after the second base from the N-terminus, but occasionally also after the first base (e.g., in short neuropeptide F (sNPF) (Predel *et al.*, 2010)). Only a small number of peptides are cleaved at a true monobasic site containing only an R or a K (Fricker, 2012). Enzymatic cleavage leaves one or two basic amino acids at the C-terminus, which are subsequently removed by a carboxypeptidase (Fricker, 2012). Most neuropeptides are amidated at the C-terminus, a modification necessary for receptor recognition. Amidation is a two-step process, in which a C-terminal glycine is hydrolysed and subsequently cleaved (Kolhekar *et al.*, 1997; Prigge *et al.*, 2000).

5.3 G-Protein coupled receptors (GPCRs)

Most neuropeptides act through GPCRs, the largest gene family of receptors (Fredriksson *et al.*, 2003). Most GPCRs that bind neuropeptides are grouped in the rhodopsin-like GPCR family (subgroup β or γ) and fewer are grouped in the secretin-like class (Fredriksson *et al.*, 2003). GPCRs typically have seven membrane spanning α-helices (transmembrane domains), each comprising approximately 20-30 hydrophobic amino acids that are connected via extra- and intracellular loops. The N-terminus is located in the extracellular space and

usually contains several glycosylation sites. The C-terminus is located in the cytoplasm and holds potential phosphorylation sites. The ligands of the receptor can bind to a ligand binding pocket, which is formed by the extracellular domains and parts of the transmembrane domains. The intracellular parts facilitate interaction with a member of the heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins), which consist of α-, β- and γ-subunits (Bockaert & Pin, 1999).

Ligand binding induces a conformational change in the GPCR, which activates the coupled G-protein by promoting release of guanosine diphosphate (GDP) from the α-subunit. Subsequently, the α-subunit binds a GTP molecule, resulting in the dissociation from the βγ subunits, which releases both complexes from the receptor. These subunits then elicit intracellular responses through the action of various signalling cascades, until activity of the Gα is stopped by hydrolysis of the bound GTP to GDP (Fricker, 2012). There are several subfamilies of the α-subunit, each containing multiple members that signal through different pathways. The most common ones are $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i/o}$. The $G\alpha_q$ subunit activates phospholipase Cβ (PLCβ), which hydrolyses phospholipids into diacylglycerol (DAG) and inositol triphosphate (IP3), which then can act as second messengers. DAG activates protein kinase C (PKC), which can phosphorylate various molecules, and IP3 mobilizes Ca^{2+} from intracellular stores such as the endoplasmic reticulum. The $G\alpha_s$ subunit mediates receptor dependent activation of the membrane-integral enzyme adenylyl cyclase, which catalyses the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Increased cAMP levels stimulate other enzymatic processes, which in turn can lead to short-term effects, such as reducing the conductance of K^+ channels, or long-term effects involving gene-expression changes. An additional pathway for the $G\alpha_s$ subunit is the activation of Ca^{2+} channels. In contrast to $G\alpha_s$, the $G\alpha_{i/o}$ subunit inhibits adenylyl cyclase leading to a decrease in cAMP concentration within the cell. Activating a receptor coupled to the $G\alpha_{i/o}$ subunit can also activate K^+ channels (Wettschureck & Offermanns, 2005). The coupling of neuropeptide GPCRs to different Gα-subunits can activate various pathways, with sometimes opposite effects in the cell (Wettschureck & Offermanns, 2005). This makes it difficult to predict the function of neuropeptide signalling in a network, based only on knowledge of the receptor location. Additionally, GPCRs are not always faithful to one specific subunit (Meeusen *et al.*, 2003; Huang *et al.*, 2014), increasing the complexity of neuromodulatory functions.

In the pre-genomic era, it was very difficult to characterize a specific GPCR and couple it to its cognate ligand (Caers *et al.*, 2012). However, with the publication of the *D. melanogaster* genome (Adams *et al.*, 2000), followed by

the release of genomes of several other insects (Yin *et al.*, 2016), it has become possible to predict the structure of a GPCR based on available genomic data (Hewes, 2001; Riehle *et al.*, 2002; Hauser *et al.*, 2008). Since then, considerable progress has been made in the deorphanization of a variety of neuropeptide GPCRs in *D. melanogaster* and other insects (Caers *et al.*, 2012).

Current methods for GPCR characterization include a reverse pharmacological approach, in which a gene encoding neuropeptide receptor is identified in the genome and expressed in a heterologous system (Caers *et al.*, 2012). The most commonly used expression system is Chinese hamster ovary (CHO) cells, but human embryonic kidney 293 (HEK) cells and *Xenopus levis* oocytes are also used (Caers *et al.*, 2012). Irrespective of expression system, the GPCRs are expressed and then exposed to potential ligands, whereupon receptor activation is measured based on the visualization of an intracellular response. As mentioned above, GPCR receptors can couple to several G-protein subunits, and for most GPCRs the specific G-protein is unknown. To circumvent this problem, several cell assays make use of the promiscuous G protein α-subunit $G_{\alpha 16}$, which couples with most GPCRs, independent of the natural G protein preference. The $G_{\alpha 16}$ subunit redirects the intracellular response towards the release of Ca^{2+} via activation of PLC (Offermanns and Simon, 1995). Intracellular Ca^{2+} can be measured using, for example, a bioluminescence- or fluorescence-based assay. In the bioluminescence-based assay, the interaction of the bioluminescent protein aequorin with calcium is used, which leads to the emission of light (Staubli *et al.*, 2002; Hauser *et al.*, 2006). For the fluorescence-based assay, a calcium-sensitive fluorophore is used (Bender *et al.*, 2002). In another type of assay, intracellular cAMP is measured using a reporter plasmid, which has a cAMP response element that transcribes luciferase (Hearn *et al.*, 2002; Johnson, 2004). When using *Xenopus* oocytes as a heterologous expression system, receptors are usually characterized by electrophysiological recording. In this assay the GPCR is expressed together with inwardly rectifying potassium channels. Upon ligand binding, inward K^+ currents can be measured (Kofuji *et al.*, 1995; Ho & Murrell-Lagnado, 1999; Ulens *et al.*, 1999). Each of the described heterologous systems comes with their own advantages; for example, the possibility of circumventing an unknown G-protein pathway, or the desire to test ligands in a high-throughput screen, makes one or the other of the systems preferable, depending on the research question.

Apart from the identification of the cognate neuropeptide of a specific receptor, characterization of a GPCR can still hold surprises. For example, it was shown that the sNPF receptor in the fire ant *Solenopsis invicta* detects a modified sNPF, which is more similar to the neuropeptide F (NPF), a feature

so far unique in insects (Bajracharya *et al.*, 2014). Additionally, many neuropeptide isoforms can bind to their cognate GPCR with different affinities as seen e.g., in the allatostatin-A family (Verlinden *et al.*, 2015). This indicates the importance of receptor characterization for all neuropeptide isoforms and homologues of known receptors.

5.4 Neuropeptide families in the regulation of feeding behaviour

Neuropeptides can have multitudes of functions by acting at different locations and in response to different internal signalling cascades. In addition, a single neuropeptide family can be involved in diverse physiological processes and behaviours (Nässel & Winther, 2010; Schoofs *et al.*, 2017). Moreover, a complex behaviour like foraging may involve several neuropeptides, working in concert (Itskov & Ribeiro, 2013; Schoofs *et al.*, 2017). The topic of this thesis is the regulation of host-seeking, which is dependent on the stage within the gonotrophic cycle, but also the nutritional state of the mosquito (Klowden, 1990). So far, only limited information is available about the neuropeptidergic regulation of host-seeking. Hence, in the following paragraphs, I will introduce the major neuropeptide families that have been described to play a significant role in regulating feeding behaviour in other insects, with a focus on *D. melanogaster*. The information about neuropeptides shown to be involved in host-seeking in mosquitoes will be discussed in more detail in chapter *5.6*.

5.4.1 Tachykinin (TK)

The TK-like peptide family is ancient and conserved, and orthologous with the vertebrate TKs (Van Loy *et al.*, 2010). The TK precursor is conserved across insects and carries up to six isoforms with a conserved C-terminal motif of FxGxRamide (Van Loy *et al.*, 2010; Vogel *et al.*, 2013). Also, the TK receptor is conserved across insects, including mosquitoes, and has been characterized in for example in *D. melanogaster* (Birse *et al.*, 2006; Van Loy *et al.*, 2010; Vogel *et al.*, 2013, 2015) Tachykinin and its receptor are expressed in interneurons in most neuropils of the central nervous system (CNS) and endocrine cells in the midgut of insects (Nässel, 2002; Wegener & Veenstra, 2015). In the peripheral and primary olfactory systems, TK signalling has been shown in the antennae (Meola *et al.*, 1998; Meola & Sittertz-Bhatkar, 2002; Jung *et al.*, 2013; Gui *et al.*, 2017). and the antennal lobe (Nässel, 2002; Carlsson *et al.*, 2010; Siju *et al.*, 2014). While immunoreactivity to TK has been shown in the antennae of both *Culex salinarius* and *Ae. aegypti* (Meola *et*

al., 1998; Meola & Sittertz-Bhatkar, 2002), these results have not been supported in later studies (Siju *et al.*, 2014). A more conserved characteristic of TK is the expression in the AL. In all insects studied so far, is its co-expression with GABA in LNs (Nässel, 2002). In *Ae. aegypti*, seven to nine LNs with cell bodies lateral to the AL are found to express the neuropeptide, while in *D. melanogaster* ca. 20 LNs, distributed in two cell clusters, are found (Carlsson *et al.*, 2010; Siju *et al.*, 2014). In *D. melanogaster*, the TK receptor is expressed in LNs and OSNs (Ignell *et al.*, 2009).

Expression of TK in the antennae of insects suggests that TK modulates antennal sensitivity (Jung *et al.*, 2013; Gui *et al.*, 2017). In *P. americana*, injection of TK reduces olfactory responses (Jung *et al.*, 2013), while in the oriental fruit fly *Bactrocera dorsalis*, the silencing of either the TK-precursor or its receptor reduces the antennal response, suggesting an opposite role for TK in this insect (Gui *et al.*, 2017). In the *D. melanogaster* AL, TK is an important regulator of foraging behaviour. Tachykinin modifies olfactory sensitivity and innate odour preference, and appears to be linked with repellent responses to high concentrations of specific odours (Winther *et al.*, 2006; Ignell *et al.*, 2009). The regulation of olfactory behaviour by TK within the AL will be discussed in more detail in chapter *5.5*.

5.4.2 Short neuropeptide F (sNPF)

The short neuropeptide F family has only been found in arthropods (Nässel & Wegener, 2011). A single sNPF precursor has been identified in all insects studied so far, including a variable number of sNPF isoforms, ranging from one to two in non-dipteran species and four to five in dipterans (Nässel & Wegener, 2011). The sNPF C-terminal consensus sequence is xPxLRLRFamide, which for some isoforms in dipterans, including mosquitoes, has been modified to xPxRLRWamide (Nässel & Wegener, 2011). In *Ae. aegypti*, a duplication event appears to have yielded a second sNPF-like gene, which encodes the *Aedes*-Head Peptides (HPs) (Matsumoto *et al.*, 1989; Stracker *et al.*, 2002; Nässel & Wegener, 2011). This duplication event appears to be species-specific as no similar gene has been discovered in any other arthropod (Nässel & Wegener, 2011). The HP precursor yields three copies of the neuropeptide, which after post-translational modifications is of the sequence pERPhPSLKTRFa (with pE being a pyroglutamic acid and hP being a hydroxyproline) (Stracker *et al.*, 2002).

The sNPF receptor has been identified as a single-copy gene in *D. melanogaster* and *An. coluzzii*. These receptors and orthologues in several other insects have been described to be highly sensitive to sNPF (Garczynski *et*

al., 2006, 2007; Yamanaka *et al.*, 2008; Dillen *et al.*, 2013; Bajracharya *et al.*, 2014; Caers *et al.*, 2016b). In *Ae. aegypti*, Liesch *et al.* (2013) identified one receptor (NPYLR1), which responds to all endogenous sNPF isoforms with high sensitivity and to HP with lower sensitivity. The high sequence similarity between *Ae. aegypti* NPYLR1 and the characterized sNPF receptor in *An. coluzzii* suggests that NPYLR1 is an orthologue sNPF receptor (Garczynski *et al.*, 2007; Liesch *et al.*, 2013). So far NPYLR1 is the only receptor found to be responsive to HPs.

Short neuropeptide F is a pleiotropic peptide, which in *D. melanogaster* shows a wide distribution in a large number of neurons and neurosecretory cells in the CNS (Nässel & Wegener, 2011). In both *D. melanogaster* and *Ae. aegypti* detailed studies have been conducted on sNPF immune-positive neurons innervating the AL, revealing expression in different types of AL neurons (Carlsson *et al.*, 2010; Siju *et al.*, 2014). In *Ae. aegypti*, sNPF is expressed in 4 to 10 LNs innervating a subset of glomeruli (Siju *et al.*, 2014). In contrast, in *D. melanogaster* sNPF is expressed in OSNs that innervate a subset of glomeruli (Carlsson *et al.*, 2010). Expression of sNPF has also been found in the antenna in another dipteran species, the oriental fruit fly *B. dorsalis* (Jiang *et al.*, 2017). The different expression of sNPF in the olfactory system of *Ae. aegypti* and *D. melanogaster* suggests divergent functions in the two species.

Short neuropeptide F has been associated with various physiological processes, of which a key function that is conserved throughout arthropods is the regulation of feeding behaviour (Nässel & Wegener, 2011). In various insects, the sNPF system is either upregulated upon starvation or positively linked to increased food intake, including the flies *D. melanogaster* (Lee *et al.*, 2004; Root *et al.*, 2011) and *B. dorsalis* (Jiang *et al.*, 2017), the honey bee *Apis mellifera* (Ament *et al.*, 2011), the cockroach *P. americana* (Mikani *et al.*, 2012), and the moth *Bombyx mori* (Nagata *et al.*, 2012). In other insects, starvation leads to a downregulation of the sNPF system or is negatively linked to food intake, as observed in *S. invicta* (Chen & Pietrantonio, 2006), *Ae. aegypti* (Liesch *et al.*, 2013) or *Schistocerca gregaria* (Dillen *et al.*, 2013, 2014). There appears to be no evolutionary trend explaining whether sNPF acts orexigenically or anorexigenically in a certain insect. Interestingly, in both *Ae. aegypti* and *S. invicta*, sNPF or its receptor are expressed in LNs, opposite to the expression in OSNs in *D. melanogaster* and *B. dorsalis*, which suggests a potential link between the AL neuron type and function (Castillo & Pietrantonio, 2013; Siju *et al.*, 2014). One mechanism involving sNPF in feeding regulation linked to the olfactory system has been described for *D. melanogaster*, which will be discussed in more detail below *5.5*.

5.4.3 Allatostatin-A (AstA)

Allatostatin-A is named after its first discovered function in the cockroach *Diploptera punctata*; the inhibition of juvenile hormone biosynthesis (Woodhead *et al.*, 1989). The name, however, is misleading, as AstA is not necessarily allatostatic, but may have other functions (Verlinden *et al.*, 2015). The single AstA precursor contains a number of isoforms, highly variable between insect species, that are characterized by a conserved pentapeptide C-terminal sequence (Y/F)xFG(L/I)-amide (Bendena *et al.*, 1999). In mosquitoes, five AstA isoforms are found (Bendena *et al.*, 1999). Allatostatin-A is detected by two receptors, AstAR1 and AstAR2, in dipterans (Félix *et al.*, 2015). The duplication of the AstA receptors seem to be an dipteran-specific event, as only one receptor has been found in other insects (Verlinden *et al.*, 2015). In mosquitoes, the AstA receptors have been characterized in *An. coluzzii* by Félix *et al.* (2015) and in *Ae. aegypti* and *Cx. quinquefasciatus* as part of this thesis work.

Allatostatin-A in *D. melanogaster* and *Ae. aegypti* is expressed in the CNS in several cell groups consisting of two or six cells in the proto- and tritocerebrum, as well as in several interneurons innervating the optical lobes, the central complex and the AL, as well as being expressed in the enteroendocrine cells in the midgut (Yoon & Stay, 1995; Hernández-Martínez *et al.*, 2005; Carlsson *et al.*, 2010; Siju *et al.*, 2014). While AstA is expressed in LNs of both *Ae. aegypti* and *D. melanogaster*, the number of LNs differs between species, with 12-16 LNs innervating a subset of glomeruli in *Ae. aegypti* (Siju *et al.*, 2014) whereas only three LNs are found in *D. melanogaster* (Carlsson *et al.*, 2010). The distribution of AstA in neurosecretory cells, as well as in local neurons, suggests that AstA can function as paracrine released factor or confined to a local network.

Allatostatin-A has been described to act directly as a regulator of foraging behaviour. In *D. melanogaster* larvae, the knock-down of either the AstAR1 or the AstA precursor reduces foraging behaviour in the presence of food (Wang *et al.*, 2012). Moreover, Hergarden *et al.* (2012) demonstrated that the constitutive activation of AstA-expressing cells in adult *D. melanogaster* decreases starvation-induced feeding behaviour, while constitutive inactivation of these AstA cells under restricted food conditions increased feeding. The observed decrease in feeding behaviour induced by AstA activation can be reversed by simultaneously activating NPF-expressing neurons, suggesting that the two neuropeptides may act antagonistically to control feeding in *D.*

melanogaster (Hergarden *et al.*, 2012). A recent study further investigated the mechanism underlying feeding inhibition in *D. melanogaster*, and showed that the conditional activation of a subset of AstA neurons, which have cell bodies in the posterior lateral protocerebrum, and enteroendocrine cells in the posterior midgut, are sufficient to reduce food intake (Chen *et al.*, 2016). This raises the possibility that AstA modulation in the CNS is at least partly mediated by neurohormonal release from enteroendocrine cells in the midgut (Wegener & Veenstra, 2015; Chen *et al.*, 2016).

Allatostatin-A signalling in *D. melanogaster* appears to be dependent on the nutritional state of the animal. This was shown by measuring transcript expression of the *AstA* precursor and the *AstAR2* in response to different diets (Hentze *et al.*, 2015). Both *AstA* and *AstAR2* transcripts were downregulated after nutrient restriction, as compared to control flies fed on sucrose-rich food (Hentze *et al.*, 2015). When flies were re-fed after a period of nutrient restriction on a carbohydrate-rich diet, *AstA* and *AstAR2* transcript levels were strongly upregulated compared to those observed in nutrient-restricted flies and *ad libitum* fed control flies. When flies, however, were refed on a protein-rich diet, only a weak upregulation in *AstA* was observed (Hentze *et al.*, 2015). Furthermore, constitutive activation of AstA changed the preference of *D. melanogaster* for protein at the expense of their natural preference for sucrose (Hentze *et al.*, 2015). Interestingly, this change in preference was stronger in females compared to males, which might be an adaptation for the different reproductive requirements for protein and sugar (Hentze *et al.*, 2015). Hentze *et al.* (2015) further showed that there is a strong link between AstA signalling and both insulin and adipokinetic hormone expression. This suggests that AstA can act as a nutrient sensor which will be discussed in chapter *5.4.5*.

5.4.4 Neuropeptide F (NPF)

Despite the name, and a similar consensus sequence, NPF is not related to the insect sNPF, although they overlap in function (Nässel & Wegener, 2011). In most invertebrates, one or two genes encode NPF-like precursors invariably containing a single copy of NPF (Nässel & Wegener, 2011). The NPF neuropeptide is comparatively long with more than 28 amino-acid residues, of which several are conserved in invertebrates, including a C-terminal motif of RxRF (mostly RPRFa or RVRF) (Nässel & Wegener, 2011). The NPF precursors have been characterized in a number of species, including *D. melanogaster*, *Ae. aegypti*, and *An. coluzzii* (Brown *et al.*, 1999; Stanek *et al.*, 2002; Garczynski *et al.*, 2005; Nässel & Wegener, 2011). The NPF receptor has been predicted in several insect species (Caers *et al.*, 2012) and has been

characterized in, for example, *D. melanogaster* and *An. coluzzii* (Garczynski *et al.*, 2002, 2005; Deng *et al.*, 2014; Caers *et al.*, 2016a). In *Ae. aegypti* the NPF-like receptor NPYLR8 was identified as a likely orthologue to the characterized NPF receptors (Liesch *et al.*, 2013).

In *D. melanogaster*, a large number of NPF-expressing neurons have been detected in the CNS, as well as in endocrine cells in the midgut (Nässel & Wegener, 2011). Interestingly, all NPF neurons in the CNS of *D. melanogaster* are interneurons (Nässel & Wegener, 2011). Neuropeptide F expression in the CNS of *Ae. aegypti*, on the other hand, appears to be restricted to one pair of medial neurosecretory cells, up to 6 other pairs of cells in the protocerebrum, and one pair of cells in the subesophageal ganglion. Additional cells are found in a ring in the cardia region of the midgut and the posterior midgut. High titres of the neuropeptide are found circulating in the lymph suggesting a neurohormonal release of this neuropeptide in *Ae. aegypti* (Stanek *et al.*, 2002). While NPF is expressed in neurosecretory cells in the CNS of *Ae. aegypti* and other insects (Nässel & Wegener, 2011) this is not the case in *D. melanogaster*, suggesting a differential neurohormonal role of NPF in insects.

Neuropeptide F has been associated with the regulation of feeding in many insects (Nässel & Wegener, 2011; Schoofs et al., 2017). In *D. melanogaster*, a clear orexigenic function of NPF has been observed. Larvae of *D. melanogaster* are highly attracted to food prior to pupation, during which state they exhibit food aversion (Wu *et al.*, 2003). These state-dependent changes in behaviour are correlated with changes in *NPF* gene expression, which is highly upregulated in larvae that are attracted to food, but downregulated in larvae that exhibit food aversion (Wu *et al.*, 2003). Overexpression of NPF in larvae prolongs the time during which the larvae are feeding (Wu *et al.*, 2003). Moreover, NPF signalling increases the acceptance of resources and conditions that are otherwise avoided (Wu *et al.*, 2005a; b; Lingo *et al.*, 2007). For example, the overexpression of NPF increases the larval tolerance for noxious food and/or under less preferable environmental conditions such as cold temperatures, whereas knock-down of NPF signalling reverses these phenotypes (Wu *et al.*, 2005a; b; Lingo *et al.*, 2007).

Studies in *D. melanogaster* suggest that several NPF-associated neuronal pathways in the CNS are involved in the regulation of fly feeding. For example, Wang *et al.*, (2013) was able to show that the disruption of NPF signalling blocks odour-mediated feeding behaviour by causing deficits in higher-order olfactory processing (Wang *et al.*, 2013). Moreover, in a study measuring the activity of NPF neurosecretory cells in adult *D. melanogaster*, Beshel & Zhong (2013) found a strong correlation between NPF activity and the resulting behavioural attractiveness of food-related odours. *Drosophila*

melanogaster generally exhibits an increased attraction towards food-related odours during starvation, which is reflected in NPF activity. However, the correlation was also observed in fed flies, suggesting that NPF does not solely act as a hunger signal (Beshel & Zhong, 2013). These studies, in *D. melanogaster*, demonstrate that NPF signalling regulates odour-mediated feeding behaviour, probably via interneurons in the CNS (Nässel & Wegener, 2011). The involvement of NPF signalling in the modulation of feeding is also suggested in several other insects, in which NPF is found in neurosecretory cells in the CNS (Nässel & Wegener, 2011). While the involvement of NPF in feeding behaviour appears to be conserved among insects (Nässel & Wegener, 2011), it merits further investigation; particularly whether potential neurohormonal release of NPF has a functional role in odour mediated feeding behaviour.

5.4.5 Insulin like peptides (ILPs)

The insect insulin-like peptides (ILPs) are a class of neurohormones that have been associated with feeding, and which both modulate, and are modulated by, several neuropeptides (Nässel & Broeck, 2016). In insects, a varying number of partly functionally redundant ILPs exists (eight in *Ae. aegypti*, seven in *An. coluzzii* and eight in *D. melanogaster)*, while only one insulin receptor is known (Krieger *et al.*, 2004; Riehle *et al.*, 2006; Nässel *et al.*, 2013, 2015; Nässel & Broeck, 2016). In the CNS of adult *D. melanogaster*, ILPs are expressed by a set of 14 median neurosecretory cells, also termed insulin-producing cells (IPCs), which have distinct arborisations in the pars intercerebralis, the tritocerebrum and several other parts of the body (Nässel *et al.*, 2015). While this pattern of expression is recapitulated in mosquitoes, additional lateral neurosecretory cells expressing ILPs can be found (Riehle *et al.*, 2006; Marquez *et al.*, 2011). The regulation of feeding by ILPs has been studied in more detail in *D. melanogaster* (Nässel *et al.*, 2015; Nässel & Broeck, 2016) than in mosquitoes, in which the focus is on the regulation of ovarian development (Brown *et al.*, 2008; Wen *et al.*, 2010; Dhara *et al.*, 2013; Strand *et al.*, 2016).

An important feature of ILPs is that they can integrate nutrient information with physiological activities in *D. melanogaster* (Wu *et al.*, 2005b). In the adult, IPCs serve directly as a sensor of the nutritional state, specifically of glucose levels, through cell-autonomous glucose sensing (Park et al., 2014) and can be regulated by a variety of other neuromodulators (Nässel & Broeck, 2016). Functional evidence for this is seen in the fact that starvation directly modifies ILP transcript levels in *D. melanogaster* larvae (Ikeya *et al.*, 2002).

The global levels of ILPs released by the IPCs according to the nutritional status can also influence the activity of neural circuits and regulate behaviours (Root *et al.*, 2011; Ko *et al.*, 2015; Nässel & Broeck, 2016; Kim *et al.*, 2017). For example, ILP signalling can negatively affect NPF signalling in *D. melanogaster*, which is critical for feeding motivation, as described earlier (see chapter *5.4.4*). Consequently, high levels of ILPs stimulate food aversion in starved larvae, through regulating the activity of NPF signalling, while low levels of ILP increase feeding motivation (Wu *et al.*, 2005a; b; Lingo *et al.*, 2007). Another example of how ILPs modulate odour-mediated behaviour will be discussed in an example in the following chapter (*5.5*).

The IPC expresses a variety of GPCRs sensitive to biogenic amines and neuropeptides including serotonin, octopamine, TK, sNPF, and AstA and can therefore be influenced by various signalling systems (Hentze *et al.*, 2015; Nässel *et al.*, 2015; Nässel & Broeck, 2016). One distinct population of neurons innervating the IPCs are the dorsolateral peptidergic neurons (DLPs) that are potentially involved in nutrient sensing (Kapan et al., 2012). The DLPs express a gustatory receptor that responds to circulating fructose, as well as GPCRs for two diuretic hormones and AstA (Johnson, 2005; Miyamoto *et al.*, 2012; Nässel *et al.*, 2015). Moreover, the DLPs co-express sNPF and Corazonin (another neuropeptide), and knock-down of sNPF in these cells leads to a decrease in ILP transcripts in the IPCs, suggesting that sNPF is important for ILP regulation (Kapan et al., 2012). The neuropeptide AstA may indirectly regulate the IPCs through the DLPs, and AstA can influence ILPs directly. The *Drosophila*-AstAR2 is expressed in the IPCs, which are directly innervated by AstA-positive neurons, and the IPCs may also be targeted by circulating AstA produced by enteroendocrine cells from the midgut (Hentze et al., 2015). Activation of AstA-positive neurons stimulates ILP production, while a loss of function mutant has the opposite phenotype. Note, however that not only IPCs are targeted by AstA, but also cells producing the neuropeptide adipokinetic hormone, which can also regulate activity in the IPCs, suggesting that AstA regulation of the ILPs is complex. This suggests that neurohormones can be a bridge between the state of the insect and the behaviour mediated by neuromodulation of local neuronal networks. Interestingly, both the release of the neurohormone and its subsequent modulation of networks, involve interactions with other neuromodulators, emphasising the complexity of state-dependent regulation of behaviour.

5.5 Neuropeptides in nutritional state-dependent regulation of the olfactory system

Several neuropeptide families are involved in the regulation of a complex process like the state-dependent regulation of feeding behaviour. This state-dependent neuromodulation occurs at various levels within the olfactory pathway, from the change in sensitivity at the peripheral level, through altering the response profile in AL glomeruli, and on to modulation of the processes in higher brain centres. At the level of local networks, several neuropeptides work in concert to achieve an appropriate behavioural output. In *D. melanogaster*, substantial progress has been made in unravelling the molecules and mechanisms through which the response of the olfactory system is modulated to facilitate feeding. Here, the example provided in the fly will be used to demonstrate the complexity the neuromodulation of the AL circuity.

Like all insects, *D. melanogaster* shows a nutritional-state-dependent change of odour-mediated foraging behaviour. The attractiveness of food-related odours is reduced during satiety, which discourages the fly from further feeding (Root *et al.*, 2011; Ko *et al.*, 2015). Conversely, starvation increases not only the innate preference to specific food-related odours, but also the tolerance of innately aversive odours such as those emitted by low-quality food (Ko *et al.*, 2015). Several studies have shown that a variety of neuropeptides act upon the AL network to modulate the response at various levels of the system.

In a series of experiments, Ko *et al.* (2015) demonstrated how starvation modulates feeding by inducing a shift in the AL chemotopic map. The behavioural change, which leads to an increased attraction to the smell of the food-related odour of vinegar, requires the modulation of the neural circuitry within two AL glomeruli. More specifically, it requires the facilitation of synaptic outputs from the DM1 glomerulus and the suppression of those from the DM5 glomerulus. The DM1 and DM5 glomeruli were previously shown to mediate odour-guided attraction and aversion behaviours, respectively. Neuromodulators effect changes in these odour channels by modulating the action of two neuropeptides, sNPF and TK (Ko *et al.*, 2015). In the DM1 glomerulus, sNPF facilitates attraction of hungry flies by autocrine release from the OSN, hypothetically at the OSN-PN synapse. In parallel, DM5 activity is suppressed by TK released from LNs, which acts on TK-receptors on the DM5 OSNs. In this example, both neuromodulators are themselves regulated by circulating ILPs. In fed flies, ILP levels are elevated, and the sNPF and TK receptors are downregulated, while in hungry flies the upregulation of both receptors is observed.

In a recent study, the neuropeptide SIFa also modulates the AL network in a nutritional-state-dependent manner (Martelli *et al.*, 2017). SIFamide is expressed in four distinct and conserved cells in the pars intercerebralis, which arborize in most parts of the CNS, including the AL (Terhzaz *et al.*, 2007). Upon starvation, increased neuronal activity in the SIFa neurons induces activity in specific LN populations and increases the odour response in distinct PNs (Martelli *et al.*, 2017). It appears that SIFa specifically regulates the sensitivity towards appetitive odours. The SIFa neurons themselves receive input from, and are therefore likely regulated by, several orexigenic and anorexigenic neuropeptidergic neurons, known to respond to the nutritional state of the fly (Martelli *et al.*, 2017).

To further demonstrate the complexity of nutritional-state dependent regulation of olfactory-driven feeding behaviour in the fly, another neuropeptide, CCHamide-1 (Farhan *et al.*, 2013), has been described as a feeding-related brain-gut neuropeptide in *D. melanogaster* (Ida *et al.*, 2012). The CCHamide-1 receptor is expressed in OSNs, with a range broader than that of the sNPF receptor (Farhan *et al.*, 2013). Starvation induces increased activity in several OSN populations, expressing ORs and IRs, by release of CCHamide-1 in the brain (Farhan *et al.*, 2013). While the mechanism behind this CCHamide-1-induced sensitisation is not clear, the authors suggest that CCHamide-1 signalling might affect OSN sensitivity at the antennal level rather than in the AL (Farhan *et al.*, 2013).

In summary, the primary olfactory processing centre responds to metabolic signals by shaping neuronal transmission within the AL network. Upon starvation, several neuropeptides are recruited through the action of neurohormones, or by feedback through orexigenic and anorexigenic neuropeptidergic neurons, and modulate the olfactory system at various levels, including the response of OSNs and LNs. The above examples show that a state-dependent modulation can have a precise influence on specific odour channels to ultimately form an appropriate behavioural output. Nevertheless, it is not clear how well specific modulatory systems are conserved among insects. For example, a comparison of sNPF expression between *Ae. aegypti* and *D. melanogaster* revealed that sNPF is found in OSNs in *D. melanogaster* and LNs in *Ae. aegypti* (Carlsson *et al.*, 2010; Siju *et al.*, 2014). This highlights the importance of investigating behavioural neuromodulation outside of *D. melanogaster*.

5.6 Neuropeptides in blood-feeding and host-seeking behaviour in *Ae. aegypti*

As introduced in the previous chapters, blood feeding leads to a significant change in the olfactory-guided host-seeking behaviour of mosquitoes. Little is known, however, about the neuromodulators involved in regulating this behaviour. As discussed before, Klowden and colleagues showed that the second stage of blood-feeding-induced inhibition of host-seeking behaviour is mediated by a factor released by the ovaries or the fat body, but the identity of this factor remains unknown (Klowden, 1990). Consequently, we do not know whether there is direct modulation by this factor or if it recruits local networks, which thereupon modulate sensory systems, including the olfactory system. So far, only two neuropeptides, HP and sNPF, have been found to be associated with the regulation of host-seeking in *Ae. aegypti* (Brown *et al.*, 1994; Liesch *et al.*, 2013) these will be discussed here in more detail.

In an early study, Brown and colleagues found that the titre of HP was upregulated during behavioural inhibition after blood feeding, and showed that the injection of this peptide into non-blood-fed mosquitoes inhibited host-seeking behaviour (Brown *et al.*, 1994). Although these results have been reproduced (Liesch *et al.*, 2013), other studies have failed to detect HP in any female tissue (Predel *et al.*, 2010; Duvall *et al.*, 2017). The *Aedes* head peptide is, however, produced in high quantities in male accessory glands, and transferred to females only during copulation, potentially as a factor to enforce paternity (Naccarati *et al.*, 2012; Duvall *et al.*, 2017). The role of HP as the sole factor responsible for regulating the blood-meal-induced host-seeking inhibition has been rejected, as the knock-down of the *HP* gene in females does not alter host-seeking behaviour (Duvall *et al.*, 2017). These authors showed that the inhibition of host-seeking behaviour observed after the injection of HP into non-blood fed females could be mediated through the activation of the *Ae. aegypti* NPYLR1, which is activated by both HP and sNPF, a second likely candidate for the endogenously active factor (Liesch *et al.*, 2013).

The influence of NPYLR1 was investigated in detail by Liesch *et al.* (2013), who showed that the injection of sNPF-3 into the lymph of *Ae. aegypti* consistently inhibits host-seeking. Furthermore, transcript levels of *NPYLR1* are significantly upregulated in the whole body of *Ae. aegypti*, between 24 h and 72 h after a blood meal, with a peak at 48 h (Liesch *et al.*, 2013). While these observations strongly suggests that activation of the NPYLR1 receptor mediates host-seeking inhibition, the knock-out of the NPYLR1 had no detectable phenotype on neither host-seeking, feeding nor reproduction (Liesch *et al.*, 2013).

What does this mean for the regulation of host-seeking behaviour by sNPF? Despite the lack of a behavioural phenotype following NPYLR1 knock-out, it is possible that a second NPYLR1 may exist, one that mediates the inhibition of host-seeking behaviour. Liesch *et al.* however found none of the other investigated NPYLRs to respond with high sensitivity to sNPF.

Mediation of host-seeking inhibition by a single factor alone, in fact, appears unlikely. It is more likely that host-seeking behaviour is regulated through redundant signalling systems, working in concert, similar to that which was shown above (e.g. *5.5*) using *D. melanogaster* as an example. In this case, removing the sNPF signalling might not be sufficient to abolish host-seeking. Moreover, it is possible that sNPF itself is recruited by an unknown factor as part of a redundant local network, regulating host-seeking behaviour. Studies in *Ae. aegypti* and other blood-feeding insects indicate that other neuromodulatory substances are regulated in response to blood feeding (Ons, 2017). In *Ae. aegypti*, the titre of NPF decreases in blood-fed females and could mediate host-seeking by lifting an inhibition of the release of other neuromodulators (Stanek et al., 2002). This shows that more studies are necessary to understand how odour-mediated host-seeking behaviour is regulated. It remains to be answered, which humoral factors are released for example by the ovaries or fat bodies, to induce a state of behavioural inhibition. Further, the process by which this behavioural inhibition can be superseded by for example the nutritional state of the animal, merits further investigation. And lastly, while there are studies showing the expression of neuropeptides in several local networks, which of those modulators are involved in the regulation of host-seeking-behaviour remains to be determined.

6 Summary of results

Host-seeking and blood feeding are at the centre of the reproductive cycle of adult mosquitoes and have a major impact on disease transmission. This stereotypic behaviour is predominantly driven by olfactory cues and is highly state dependent. A successful blood meal leads to profound changes in the behaviour of the mosquito and in the olfactory system. It is well established that behaviour is dependent on the physiological state, realized through neuromodulation within the central nervous system. Detailed functional studies on neuromodulation of host-seeking behaviour in mosquitoes, however, are few. The aim of this PhD project was to identify the neuropeptides involved in regulating host-seeking behaviour in *Ae. aegypti*, and to characterize them in function and interaction with their receptors.

The identification of neuropeptides involved in regulating host-seeking behaviour in *Ae. aegypti* is presented in paper I. In this study, I first characterized the feeding behaviour of *Ae. aegypti* following a successful blood meal, with a focus on subsequent host-seeking but also with regard to sugar-feeding behaviour. Under laboratory conditions, host-seeking is suppressed for at least 72 h after a successful blood meal, until egg-laying. In addition, a successful blood meal inhibits sugar feeding, which gradually returns over the course of the next three days, culminating in a complete restoration in gravid mosquitoes. The focus of this study, however, was on the identification of neuropeptides involved in the regulation of the observed behaviours.

Together with my colleagues at the University of Marburg, I refined a previous method of MALDI-TOF mass spectrometry by combining direct tissue profiling with the use of isotope labelled neuropeptides. This allowed me to analyse tissues from individual female mosquitoes in a reasonable timeframe and to identify changes in neuropeptide levels depending on different feeding regimes of blood and sugar. I found that within the AL, the levels of sNPF-2, AstA-5, and NPLP-1-5 change following a blood meal, during behavioural inhibition of host-seeking behaviour.

I further injected the neuropeptides identified by the mass spectrometric analysis into non-blood-fed animals to reveal a functional link to host-seeking (Figure 1). Two of these neuropeptides, sNPF and AstA, reduced, but did not abolish, host-seeking when injected into the lymph of non-blood-fed mosquitoes. Inhibition of host-seeking behaviour to the same extend as seen in blood fed mosquitoes was only observed after the injection of a binary blend of sNPF and AstA, indicating multiple neuromodulatory systems necessary for the regulation of host-seeking behaviour. These data served as the basis for my further studies concerning the corresponding receptors for sNPF and AstA.

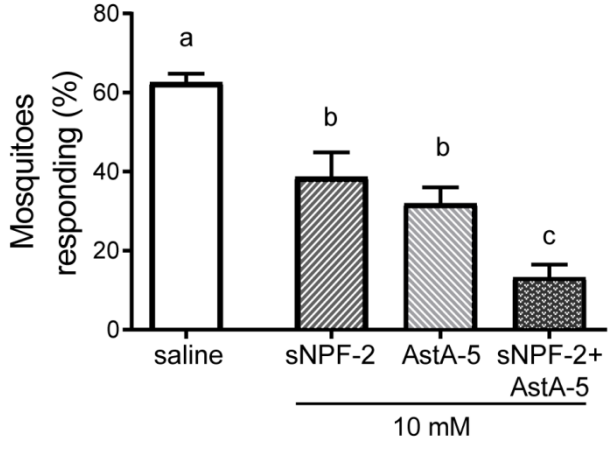

Figure 1. Systemic injection of synthetic short neuropeptide F-2 (sNPF-2) and allatostatin-A-5 (AstA-5) into non-blood fed female A. aegypti inhibits host seeking behaviour. The percentage of mosquitoes responding to human host cues after injection of physiological saline or 10 mM of sNPF-2, AstA-5, or a blend of both neuropeptides, is presented. As the behaviour of physiological saline injected animals did not differ among the replicates, the data were pooled for comparison. Six biological replicates were performed, and for each group 30-40 mosquitoes were tested. All data are plotted as mean ± SEM. Bars that are not significantly different share the same letter.

In paper II and III, I strove to extend our knowledge about the AstA and sNPF signalling systems (the neuropeptide precursors and their cognate receptors), by functionally characterizing this system in three mosquito species, *Ae. aegypti*, *An. coluzzii* and *Cx. quinquefasciatus*. Both the sNPF and the AstA signalling systems have been associated with the modulation of feeding behaviour in previous studies. This includes the stereotypic host-seeking and blood feeding in hematophagous insects. Not only the release of neuropeptides, but also the regulation of the cognate receptors can play a significant role in the regulation of behaviour.

In both papers, I first identified and cloned those neuropeptide precursors and neuropeptide receptors, which had not been described yet. I performed a comparative structural and functional characterization of the receptors by stable expression in a Chinese hamster oocyte cell line that also stably expressed a promiscuous G-protein (CHO/G_{16}). Last I conducted a quantitative real-time PCR analysis, demonstrating that transcript abundance of some of the neuropeptide receptors is regulated following feeding.

In paper II, I focused on the characterization of the AstA receptors. In *D. melanogaster* and *An. coluzzii*, two functional AstARs have been described, in contrast to other insects, where only one copy has been found. In this study, I identified, reannotated and cloned the two AstARs in *Ae. aegypti* and *Cx. quinquefasciatus.* Phylogenetic analyses of the two AstARs revealed that the mosquito AstAR1s have retained a similar amino-acid sequence as the AstARs from other insect species, in contrast to the AstAR2s. A further intron analysis, however, showed, that the number of introns accumulated in the *AstAR2* locus is similar to that in other insects, with only the final two introns being conserved across *AstAR1s* and *AstAR2s.* Functional analysis of the AstARs revealed a higher sensitivity of the AstAR2s compared to the AstAR1s in the two culicines *Ae. aegypti* and *Cx. quinquefasciatus*, while in *An. coluzzii* the two receptors displayed similar affinities (Figure 2). This indicates a divergence of the dual AstAR system between the anophelines and culicines. The quantitative real-time PCR revealed changes in the *AstAR2* transcript abundance in the heads of *Cx. quinquefasciatus*, but not the other species in response to feeding.

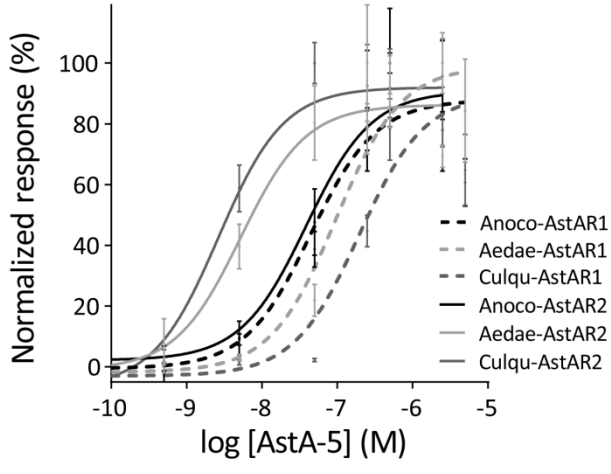

Figure 2. Functional analysis of *Anopheles coluzzii* (Anoco), *Aedes aegypti* (Aedae) and *Culex quinquefasciatus* (Culqu) allatostatin-A receptors 1 and 2 (AstAR1s and AstAR2s), in response to allatostatin-A (AstA). Dose-dependent activation of mosquito AstARs, stably expressed in CHO/G_{16} cells, and challenged with various concentrations of AstA-5 is shown. Bioluminescence was normalized to the lowest and highest values, respectively, for each replicate. The AstAR1s are indicated with dashed and AstAR2s with solid lines. Error bars represent the standard deviation.

In paper III, I characterized the sNPF signalling system in the same three mosquito species. The novelty of this study is the characterization of the sNPF signalling system in *Cx. quinquefasciatus* and its comparison with the orthologous systems of *Ae. aegypti* and *An. coluzzii*. I found several *Cx. quinquefasciatus*-specific duplications of the sNPF-3 isoform within the sNPF precursor, not reflected in the precursors of the other two species. Structural and functional characterization, however, showed that the three sNPF receptors tested all behave similarly, perhaps indicating evolutionary constraint (Figure 3). Using quantitative real-time PCR, I demonstrated that transcript abundance of the *Cx. quinquefasciatus sNPF* neuropeptide precursor and receptor is regulated following feeding.

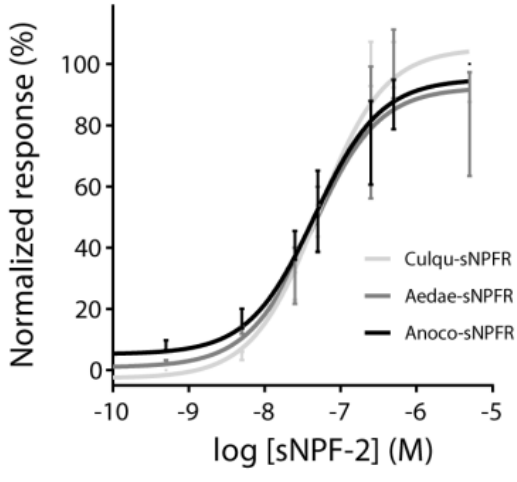

Figure 3. Functional analysis of the *Culex quinquefasciatus* (Culqu), *Aedes aegypti* (Aedae) and *Anopheles coluzzii* (Anoco) sNPFRs in response to endogenous sNPFs. The figure shows the dose-dependent activation of the sNPFRs stably expressed in CHO/G_{16} and assayed with various concentrations of sNPF-2. Bioluminescence was normalized to the lowest and highest values for each technical replicate, respectively. Error bars represent the standard deviation.

7 Conclusion and perspectives

Mosquitoes are heavily dependent on their sense of smell to locate hosts for blood feeding. An understanding of the endogenous factors regulating the odour-meditated host-seeking behaviour can lay the foundation for the development of novel methods for vector control.

In this thesis, I made considerable progress in the analysis and understanding of neuropeptidergic regulation of odour-mediated host-seeking behaviour in *Ae. aegypti*. My first paper provides evidence that the modulation of olfactory information during state dependent inhibition of host-seeking is likely regulated by at least two neuropeptides, sNPF and AstA, acting in concert in the ALs. Investigation of both the release of neuropeptides, and the function and regulation of the cognate receptors, is essential to understand the neuropeptidergic signalling system in regulation of host-seeking. The characterization of the AstA signalling system revealed the presence of a dual AstA receptor system in mosquitoes, which is divergent in the culicines, *Ae. aegypti* and *Cx. quinquefasciatus*, compared to *An. coluzzii*. The high sensitivity of the AstAR2 and its regulation in response to blood feeding in *Cx. quinquefasciatus*, makes this receptor a promising candidate as an essential component in mosquito host seeking behaviour. The AstARs in *An. coluzzii*, show similar sensitivity in line with that observed by AstAR characterization in *D. melanogaster* (Larsen *et al.*, 2001), suggesting that there has been no directional selection on ligand specificity of the two receptors. Blood and sugar feeding seemed to have no influence on transcript expression of either the neuropeptide precursors or the receptors in *Ae. aegypti* and *An. coluzzii*, while transcript levels of both the sNPFR and the AstAR change in *Cx. quinquefasciatus*. This suggests that neuromodulation in *Cx. quinquefasciatus* is regulated on the level of receptor expression, while in *Ae. aegypti* and *An. coluzzii* neuromodulation might be achieved by the release of neuropeptides. The differences between neuropeptidergic signalling systems in the three

mosquito species and *D. melanogaster* , invites to ask more in depth questions about neuropeptidergic regulation outside *D. melanogaster*.

In recent years, great progress has been made in the development of genetic tools in mosquitoes (Overcash & Adelman, 2016). The dawn of the CRISPR/Cas9 system (Jinek *et al.*, 2012; Kleinstiver *et al.*, 2016) and its establishment in mosquitoes (Gantz *et al.*, 2015; Kistler *et al.*, 2015; Hammond *et al.*, 2016) allows for site-specific gene editing, as shown for example in the recent knock-out of the *Aedes-head peptide* gene (Duvall *et al.*, 2017). Furthermore, advances have been made in establishing binary gene expression systems, for example the Q-system in *An. coluzzii* (Riabinina *et al.*, 2016). These developments can enable hypothesis driven questions concerning physiology and behaviour and could provide further insight into the regulation of host-seeking behaviour. For example, the CRISPR/Cas9 technology could be used to clarify the role of AstA signalling in regulating host-seeking in *Ae. aegypti*. More specifically, a knock-out of either the neuropeptide precursor or the high sensitive AstAR2 could provide information: first about the involvement of AstAs in host-seeking behaviour in general; and second, about the specific function of the AstARs. Moreover, the introduction of a double knock-out in the AstA and sNPF signalling systems could clarify whether these two systems are necessary to induce host-seeking inhibition in blood-fed mosquitoes. The combined power of gene editing, together with the use of the binary gene expression systems, could allow for targeted labelling of key elements within the neuropeptide signalling systems. This could reveal the expression of neuropeptide receptors in AL neuron populations. As a whole, a better understanding of the regulation of host-seeking and blood feeding in vector mosquitoes could result in the rational development of novel approaches for vector control.

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