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Diploma Thesis

Influence of the *Drosophila* Adipokinetic Hormone on Starvation Effects in Olfactory Perception

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Submitted by

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I. List of Contents

II. List of Figures

III. List of Tables

IV. List of Abbreviations

A ..... Antenna

AKH ..... Adipokinetic hormone

AKH^- ..... AKH-cell deficient flies

AL ..... Antennal lobe

BL-Nr. ..... Bloomington stock number

BS ..... Basiconic sensilla

CC ..... Corpora cardiaca

CS ..... Coeloconic sensilla

CyO ..... Balancer chromosome *CurlyO*

DAKH ..... *Drosophila* adipokinetic hormone

ETA ..... Ethyl acetate

ETB ..... Ethyl butyrate

GFP ..... green fluorescent protein

HEN 2-Heptanone

LH ..... Lateral horn

LMA ..... Low melting point agarose

LN ..... Local interneurons

MB ..... Mushroom body

MP ..... Maxilliary palp

n ..... Sample size: Quantity of tested flies

N ..... Sample size: Quantity of repeated experiments

OBPs ..... Odorant binding proteins

ORs ..... Olfactory receptors

OSNs ..... Olfactory sensory neurons

PNs ..... Projection neurons

RH ..... Relative humidity

RI ..... Response index

ROI ..... Region of interest

RPCH ..... Red pigment-concentrating hormone

rpr ..... gene *reaper* triggering apoptosis

sac ..... Sacculus

SSR ..... Single sensillum recording

T ..... Temperature

TS ..... Trichoid sensilla

UAS ..... Upstream activating sequence

WT ..... Wild-type

1 Abstract

Insects are exposed through an environment containing a wide range of different odours. Their behavioural response towards the smelling background depends on external cues as well as on the internal physiological state. The latter is, amongst others, affected by a wide range of different neuromodulators (BAUMANN & GERSCH, 1982; MILDE *et al.*, 1995; GÄDE *et al.*, 1997; VAN DER HORST *et al.*, 2001; LEE & PARK, 2004; ALTSTEIN & NÄSSL, 2010). One of these neuromodulators is the adipokinetic hormone (AKH), which is involved in a remarkable number of physiological processes. In order to examine the extent of its multifunctionality, this study investigates the hormone's role in starvation processes connected with enhanced olfactory perception. The internal state is also important for the behavioural response of the vinegar fly *Drosophila melanogaster.* For example, the circadian rhythm (KRISHNAN, *et al.*, 1999, STEPHAN 2002) and stress can influence the nervous system and alter the behaviour. ROOT *et al.* (2011) support this hypothesis on the stress factor hunger by finding an increase in food searching activities in relation to modulations of certain olfactory sensory neurons. Similar effects were studied by FARHAN *et al.* (in preparation). The research of LEE & PARK (2004) on hungry AKH-cell deficient fruit flies (AKH^{-}) underlines the role of AKH in starvation processes.

During this study, in order to examine the influence of AKH in this context, the technique of single sensillum recordings (see section 3.3.1) has been used for analysing the response of the first level of odour coding, the antenna, towards a food-related odorant (ethyl acetate). This method allows the visualization of the response of the olfactory appendage, the antenna, especially ab2A neurons of basiconic sensilla. Even on this level of odour coding there might be a dependence on the presence of AKH. To summarize, I cannot exclude the fact that AKH is involved in starvation processes related to olfaction.

I compared the physiological response of *Drosophila* with behavioural decisions towards ethyl acetate. In order to analyse this, I used a binary trap assay. Moreover, additional behavioural assays (*Flywalk*, mortality assay) were used to gather information on activity patterns and lifespans of wild-type (WT) flies and flies that lacked

AKH. With the help of these methods I confirmed earlier investigations of LEE & PARK (2004) and FARHAN *et al.* (in preparation). However, I could not draw any final conclusion regarding the influence of AKH on starvation-dependent alteration of the flies' responses to a food-related odorant, since the genetic background of AKH^{-} flies might have influenced the behaviour.

2 Introduction

2.1 Olfaction

Insects are exposed to a complex environment consisting of a wide range of diverse information. They perceive their surroundings with specialized sensory organs and have to distinguish multifarious and also highly similar inputs. On this purpose, several senses like vision, olfaction, thermoception or the sense of taste and touch have to be brought together before evoking a context-related and experience-dependent behaviour. The external world has to be translated into an internal representation for identification, evaluation and localisation of the stimulus and its relationship to the insect.

The olfactory sense of insects as a special form of chemoreception evolved already more than 500 million years ago (DETHIER, 1990). Organisms of nearly all taxa from protozoa to mammals and insects possess several olfactory systems essential for oviposition and breeding, navigation, seeking potential food sources, prevention from predators and finding mating partners (pheromone detection). These aspects are crucial for the lives of insects. In this context they are able to perceive and discriminate an enormous number of odours. Despite different morphological characteristics it is remarkable that the olfactory system is similarly evolved in invertebrates and vertebrates (HILDEBRAND & SHEPERD, 1997; EISTHEN, 2002).

2.2 *Drosophila melanogaster* as a Model System

At the beginning of the 20th century T. H. Morgan discovered the little insects as laboratory animals and performed one of the first crossing experiments using these flies. Down to the present day *Drosophila melanogaster* established to be one of the most common genetic model organisms. The animal belongs to the class of the *Insecta* (order: *Diptera*, family: *Drosophilidae*). Female flies differ from males in their length and the colour of their bodies. Their short generation time and quick growth has made *Drosophila* a popular research object. Furthermore, *Drosophila* is economically and

easily to handle. In a breeding chamber with sufficient humidity, adequate temperature and day-night cycles, flies can be bred in large amounts on a common food medium. In order to ensure that larvae can feed after hatching, female mated flies oviposit a large number of eggs on food sources. After ten to twelve days depending on temperature a new generation hatches. This process is gender-dependent. At first female flies eclose, subsequently males.

As many studies have shown over the last century, this small insect is not in any way an annoying nuisance. For many scientists it is even a genetic toolbox. Since the whole genome of *D. melanogaster* has been sequenced (ADAMS *et al.*, 2000), selective and cell-specific activation of genes is possible. Thus, flies with appropriate characteristics can be generated. Manipulating the small genome is already possible at an early stage of development. One opportunity of modification is the targeted gene expression system GAL4/UAS containing two separate components (BRAND & PERRIMON, 1993).

First, a transcription factor (GAL4) is inserted in the *Drosophila* genome and connected with a tissue- or cell-specific driver. By expression of the driver additional GAL4 is expressed. The second construct, the responder, carries an upstream activating sequence (UAS) with a GAL4-binding site and a downstream lying target gene. The presence of GAL4 initiates the transcription of UAS and the target gene. In my diploma thesis I used the cell-specific driver *dAkh*, encoding the *dAkh* promoter, which in turn regulates gene expression of the *Drosophila* adipokinetic hormone (DAKH). The target gene *reaper* (*rpr*) was also used. The two components, driver and responder, are separated into parental fly lines, so that the responder construct is transcriptionally silent without activation of the driver line. By crossing the transgenic fly strains the target gene *rpr* is expressed. *rpr* triggers programmed cell death of corpora cardiaca (CC) cells containing AKH (Fig. 1).

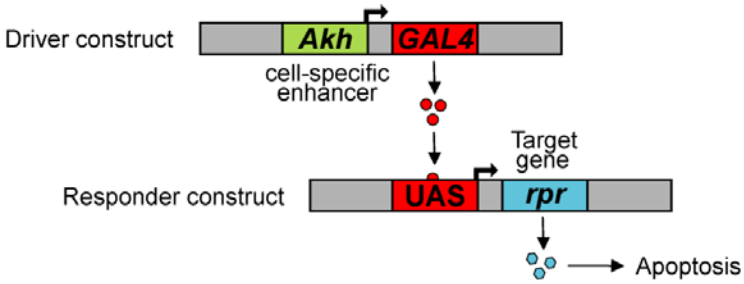

Figure 1: The Gal4/UAS-System of *D. melanogaster*.
Transcription of the cell-specific driver *Akh* activates the expression of the inserted GAL4 sequence in corpora cardiaca cells. Binding to the upstream activating sequence (UAS) in the same cells leads to expression of the target gene *rpr* is initiated. Programmed cell death is triggered.

The ability to combine the genetic toolbox with olfactory physiological and behavioural analysis *in vivo* enables to draw conclusions about the perception of the relatively simple olfactory sensory system. Fruit flies have only a small amount of olfactory sensory neurons (OSNs) as well as olfactory receptors (ORs) (CLYNE *et al.*, 1999; DE BRYNE *et al.*, 1999; ADAMS *et al*., 2000). Nevertheless, the more complex olfactory system of vertebrates and *Drosophila* resemble each other in basic structures and connectivity making it possible to compare fundamental characteristics (HILDEBRAND & SHEPERD, 1997; EISTHEN, 2002).

2.3 The Olfactory System of *Drosophila melanogaster*

Handling its everyday's life *D. melanogaster* has to deal with a wide range of odours. Therefore, flies have to filter the crucial cues out of a smelling background. The ability to process olfactory information enables them to locate and judge a food source, suitable oviposition sites, to seek mates or to protect themselves against potential predators.

Odour Detection – Antennae and Maxilliary Palps

Flies can sense odorants from the environment with special sensory appendages: the third segment of the antennae (funiculus) and the maxillary palps at the base of the proboscis. These bilaterally symmetric appendages are covered with various olfactory

hairs, termed sensilla (reviewed in LAISSUE & VOSSHALL, 2008). The sensilla are subdivided into three major morphological and functional types: The short, conical coeloconic sensilla (CS), the long and spiky trichoid sensilla (TS) and the club-shaped basiconic sensilla (BS).

The latter are able to detect mainly food-related odours in the antennae and the maxillary palps. Altogether about 220 BS with were quantified (CLYNE *et al.*, 1997; SHANBHAG *et al.*, 1999). According to their distribution on the antennal surface, their size, the characteristics of the pores and the number of containing OSNs, BS were subdivided into large and small basiconics. Either two or four neurons are housed in each large BS, whereas small BS on the antenna mostly contain two OSNs (reviewed in LAISSUE & VOSSHALL, 2008). A very short type of olfactory sensilla, the 56 CS of the antennal surface, is innervated by two or three neurons (SHANBHAG *et al.*, 1999). Coeloconics are specialized to detect water vapour, putrescine and ammonia (YAO *et al.*, 2005) as well as acids and amines like acetic acid and pyridine (SILBERING *et al.*, 2001).

There is evidence that the third type of sensilla, the trichoids, is able to respond to pheromones, but not to food-related odours. The aggregation pheromone cis-vaccenyl acetate is an identified ligand, which is able to activate *Or67d*, the OR of one subtype of TS (VAN DER GOES VAN NATERS & CARLSON, 2007; reviewed in LAISSUE & VOSSHALL, 2008). Up to three neurons are housed in each of about 150 TS (CLYNE *et al.*, 1997).

Since several types of sensilla display different distribution patterns on the surface of the funiculus, DE BRYNE *et al.* (2001) segregated them into five regions (Fig. 2). The surface of the third antennal segment bears 419 sensilla in males and 457 in females (YAO *et al.*, 2005). The sexual dimorphism of *D. melanogaster* according to the number of sensilla has already been investigated. Female flies have a longer funiculus with a larger number of BS, being important for detection of oviposition sites. Whereas in males more TS can be counted, which they use for pheromone detection of mating partners.

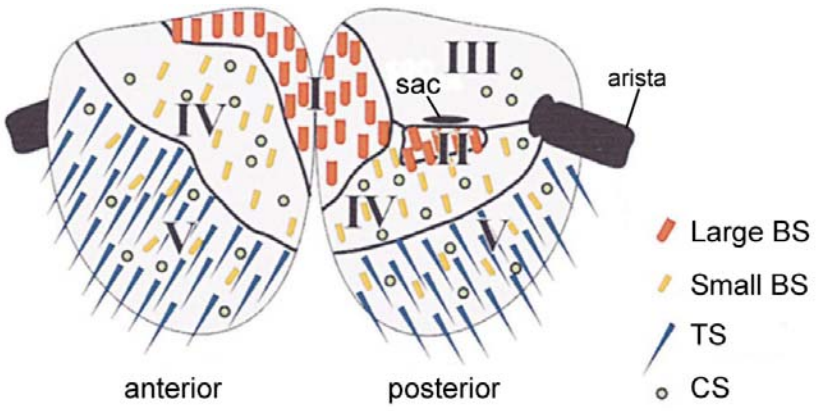

Figure 2: Distribution patterns of the different morphological types of the sensilla on the funiculus. Segregation into five spatial domains (modified from DE BRYNE *et al.* 2001). BS=basiconics sensilla, CS=coeloconic sensilla, sac=sacculus, TS=trichoid sensilla

How does the olfactory information from the environment get into the sensilla and the brain? A large number of cuticular pores perforating the sensilla allow volatiles to access (SHANBHAG *et al.*, 1999). After getting into the sensilla, it is presumed that odorants are recognized and transported by so-called specific odorant-binding proteins (OBPs) through the sensillar lymph (SHANBHAG *et al.*, 2000). Additionally, OBPs are also involved in modulations of olfactory behaviour (SWARUP *et al.*, 2011). In adult vinegar flies every type of altogether 1300 antennal OSNs (maxilliary palps only 120 OSNs) expresses selectively a small subset of one or two unique ORs. Insect ORs belong to a large family of membrane-associated proteins containing seven transmembrane domains with no homology to vertebrates´ ORs. None of the antennal specific ORs are expressed in the maxiliary palps and vice versa. In *D. melanogaster* a repertoire of 62 OR genes can be identified. ORs have selective ligand-binding properties mediating the olfactory information. More than just one odorant can activate a specific OR and more than just one OR can also respond excitatory or inhibitory to a single odorant (DE BRYNE *et al.* 2001; reviewed in LAISSUE & VOSSHALL, 2008). It is also known that a single gene, called *OrCo* (WICHER *et al.*, 2008; VOSSHALL & HANSSON, 2011), is expressed in all OSNs and dimerises with the OSN-specific ORs. The activation of the complex by an odorant leads to the depolarization of the OSNs (LARSSON *et al.*, 2004; WICHER *et al.*, 2008). Thus the *OrCo* co-receptor is necessary for the function of all ORs and the transfer of the olfactory information.

Odour Processing and Modulation – Antennal Lobe and Higher Brain Centres

The sum of all axons of OSNs together, also called the antennal nerve, project to the first processing centre of olfactory information, the antennal lobe (AL). The unique receptor activation pattern is hence represented in the brain. The AL is bilateral symmetric, which implies that odour stimulation causes the same spatial patterns of activity in each lobe. This spatial representation is generated by OSNs projecting to approximately 50 known glomeruli (IYENGAR 2010, reviewed in LAISSUE & VOSSHALL, 2008) consisting of synapses between OSNs and second-order neurons, called projection neurons (PNs). Every glomerulus gets input from a special set of OSNs expressing the same OR, which is shown in Figure 3. Accordingly, OSNs of CS, BS and TS project to different regions of the AL. The size of a glomerulus correlates directly with the number of OSNs, sending their axons to it (VOSSHALL *et al.*, 2000). In this study the response of large BS to a food-related odorant was examined. A stereotypic distribution can be observed within sensilla types meaning that every neuron of e.g. large BS possesses the same type of OR. It was already demonstrated that all ab2A neurons expressing the receptor *Or59b* project to the glomerulus DM4 (COUTO *et al.*, 2005; HALLEM & CARLSON, 2006). ab2A neurons are activated by the food-related odorant ethyl acetate I used in my experiments. It is known that ORs encoding attractive odours respond also to ethyl acetate activating the same glomeruli. (SEMMELHACK & WANG, 2009; KNADEN *et al.*, 2012; COUTO *et al.*, 2005; FISHILEVICH & VOSSHALL, 2005; HALLEM & Carlson, 2006; DE BRYNE *et al.*, 2001; GALIZIA *et al.*, 2010).

Local interneurons (LN), creating links between various glomeruli (Fig. 3), modulate the olfactory information according to their context through excitation[1] and inhibition[2], respectively, mediated by both neurotransmitters acetylcholine and γ-aminobutyric acid (WINTHER & IGNELL 2010). Beside excitatory and inhibitory neurons, additional extrinsic neurons innervate the AL. This third neuron type of the AL releases neurotransmitters like octopamine and serotonin, IPNamide and SIFamide being also neuropeptides or dimethyl sulfide. Furthermore, multiple neuropeptides contribute in the

[1] OLSEN et al., 2007; ROOT et al., 2007; SHANG 2007; HUANG et al., 2010; YAKSI et al., 2010
[2] NG *et al.*, 2002; ROOT *et al.*, 2008; OLSEN & WILSON, 2008; YAKSI *et al.*, 2010

complex modulatory circuit in order to process olfactory information resulting in a context-related behaviour (CARLSSON *et al.*, 2010).

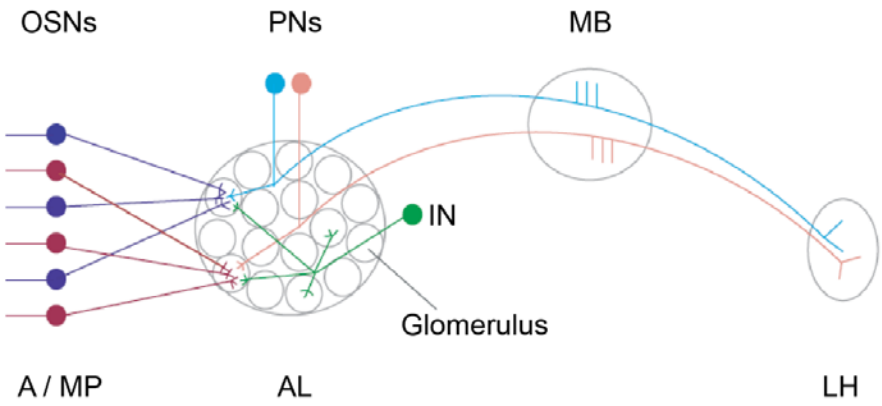

Figure 3: Relay of the olfactory system of *D. melanogaster*.
Projection and relay of OSNs and PNs into higher brain centres. A=Antenna, AL=Antennal lobe, IN=Local interneuron, LH=Lateral horn, MB=Mushroom body, MP=Maxilliary palp, OSN=Olfactory sensory neuron, PN=Projection neuron. Adapted from HALLEM & CARLSON (2004).

From the antennal lobe PNs relay olfactory information to higher brain centres like the mushroom body or the lateral horn (secondary centres) (Fig. 3). Their entire role is still not completely understood. Investigations from HEISENBERG *et al.*, (1985) demonstrated that the mushroom body is not only involved in sexual orientation and courtship behaviour in males (FERVEUR *et al.*, 1995; O'DELL *et al.*, 1995), sleep (JOINER *et al.*, 2006) and suppressing locomotor activities (MARTIN *et al.*, 2011), but is also required for olfactory learning and memory. PN axons are also sent to the later horn, thus enabling the integration of olfactory information from several AL glomeruli.

In summary, when an odour has encountered the sensilla of *D. melanogaster*, it has to be translated, processed and modulated within the complex olfactory circuit of the insect's brain. Furthermore, it has to be evaluated and compared with additional external and internal information. This leads to a context-related behaviour of the fruit fly in response to the odour and entails that every step of odour processing involved can alter the resulting behaviour.

2.4 The Influence of Starvation on Olfaction

Currently, it is not very well known, how a fly modulates its olfactory information, evaluates and discriminates an odorant or how it handles the complexity of an odour mixture resulting in its behaviour. Hypotheses about internal, mediating factors like starvation that influence olfactory circuits and the attractiveness of an odorant are investigated. *D. melanogaster* absorbs its energy from food sources, being essential for its survival. Starvation for a longer time affects the whole metabolism and leads to decreased levels of carbohydrates, lipids and proteins (MARRON *et al.*, 2003). DJAWDAN *et al.* (1997) demonstrated a lower CO_2 production in starved vinegar flies than compared to fed flies indicating an impact on the metabolism after starvation. Regarding olfaction, starved vinegar flies show increased behavioural and physiological responses to an odorant (ROOT *et al.*, 2011). Under that condition they demonstrated higher food searching activities as well as increased activities in DM1, DM2 and DM4. In order to contribute to current research in this field, possible influencing factors are examined. One of these modulators, which have to be investigated, is the AKH of *D. melanogaster*.

2.5 The *Drosophila* Adipokinetic Hormone

The group of neuropeptides play an essential role in the regulation of physiological and developmental processes both in vertebrates and invertebrates. It includes a great diversity of peptides being released from neurosecretory cells, interneurons, motoneurons or sensory neurons and effect the central or the peripheral nervous system as a neuromodulator or circulating hormone (ALTSTEIN & NÄSSL, 2010). Certain insect neuropeptides act strictly local at short distance as synaptic modulators or co-transmitter; others are released in the hemolymph and transported to more diffuse target tissue (NÄSSL, 2002). About 35 genes expressing neuropeptides in *Drosophila* are known. Their expression pattern is highly cell-specific with rare colocalization of other neuropeptides (ALTSTEIN & NÄSSL, 2010). 30-40 larger precursor proteins, also called prepropeptides, are encoded by genes process neuropeptides of different insect species.

Neuropeptides are classified in several neuropeptide families depending on sequence

homologies or functional similarities of different taxa. One large family consists of several insect neuropeptides, the AKHs and one crustacean peptide, the red pigment-concentrating hormone (RPCH) of prawn. Currently, 47 members of the so called AKH/RPCH family are known (GÄDE, 2009). Like most neuropeptides AKH/RPCH peptides are multifunctional. They are involved in a wide range of functions in different insect species. These functions include the mobilization of stored lipids and carbohydrates from the fat body during intense physical activity (e.g. flight), the stimulation of motor neurons in the moth *Manduca sexta*, cardioacceleration in the cockroach *Periplaneta americana*, and in crustaceans they are responsible for colour adaptation, to name but a few (LEE & PARK, 2004; MILDE *et al.*, 1995; BAUMANN & GERSCH, 1982; GÄDE *et al.*, 1997). It is also known that AKHs act as functional homologs of the vertebrate glucagon, which regulate blood sugar levels (VAN DER HORST *et al.*, 2001). In insects the disaccharide trehalose is the major sugar molecule in the hemolymph. AKHs raise the trehalose in the hemolymph of several insects and can hereby control the sugar homeostasis (VAN DER HORST *et al.*, 2001).

In adult *Drosophila melanogaster dAkh* is expressed in about 13 neurosecretory cells of the paired corpora cardiaca (CC), which is the main neurohemal organ of the endocrine system closely associated with the brain (NÄSSL, 2002; GÄDE *et al.*, 1997). The stimulation of the AKH-producing CC cells of the ring gland by hypoglycemia raises the intracellular calcium level of AKH-producing cells and leads to exocytose of the neuropeptide at the axon terminals (VAN DER HORST *et al.*, 1999; HASELTON & FRIDELL, 2010. Depending on the developmental stage of a fly the target tissues of the DAKH are the prothoracic gland and the aorta in larval stages whereas in adults it is the brain and the crop (LEE & PARK, 2004). Even in vinegar flies they mediate the mobilization of carbohydrate from the fat body and in higher doses they cause cardioacceleration in prepupae (NOYES *et al.*, 1995).

The inactivation of *a* neuropeptide-mediated signal is realized both by enzymatic degradation of the peptide and by desensitization of the receptor (NÄSSL, 2002).

2.6 Aims of this Study

The previously named findings lead to the assumption that the AKH plays a role in starvation processes. Recent studies on this topic have demonstrated that reduced insulin signaling in relation to expressions of the receptor of short neuropeptide F (sNPFR1) act as modulators in this neural circuit (ROOT *et al.*, 2011). Nevertheless, they demonstrated this effect only in specific OSNs (Or42b, Or59b and Or22a neurons), leading to the assumption there might be more being involved in it. Currently, it is not well understood, how the altering of behaviour and neural circuits is modulated. In *Drosophila* insulin-like peptides act as antagonists of AKH. Both are involved in hemolymph sugar homeostasis and starvation processes. Consequently, other modulators might play an important role, so DAKH is a predestinated neuropeptide to have a closer look at. It is associated with processes of starvation, while raising the locomotory activity. Additionally, there is evidence that flies without AKH-cells exhibit a strong resistance to starvation-induced death (LEE & PARK, 2004). Based on its multiple functions in insects olfactory perception of hungry fruit flies possibly depends on DAKH. Therefore, the aims of this study are the following:

- Comparing the physiological response of ab2A neurons to a food-related odorant in AKH^- flies and WT flies under starvation conditions.
- Comparing behavioural decisions of AKH^- flies and WT flies in a binary trap assay after different durations of starvation.
- Examining the activity and lifespan of AKH^- flies.

3 Material and Methods

3.1 Fly Lines and Rearing

For my studies I used *Drosophila melanogaster* vinegar flies at an age of 4 - 5 days. Flies were raised on conventional cornmeal medium (Tab. 1, Fig. 4A) at 25°C, 70% relative humidity and a 12 hours day-night cycle. Every three days I transferred the flies into a fresh vial containing the food medium.

Table 1: Contents of two fly food media.

Cornmeal Medium (1 l) LEWIS, 1960	**Thorpe´s Medium (1 l)** THORPE *et al.*, 1939; CHAKRABORTY *et al.*, 2009
918 ml water 118 g treacle 11 g beer yeast 4,1 g Agar-Agar 95 g cornmeal (polenta) 2,4 ml propionic acid (99%) 3,3 ml Nipagin (Methyl-4-hydroxy-benzoate 30%)	1000 ml water 8 g Potassium sodium tartrade tetrahydrat 2 g Ammonium sulphate ($(NH_4)2SO_4$) 5 g L(+)Tartaric acid cryst 0,65 g Magnesium sulphate 0,5 g Potassium dihydrogen orthophosphat 0,25 g Calcium chloride dihydrat 20 g Agar (4%) 7,5 g Sucrose (1,5%)

Eclosed animals were transferred for four days on an artificial, nearly odour-free medium (Thorpe's medium) in order to test olfactory responses of naive flies and to prevent adaptation, experience and learning effects (IYENGAR *et al.,* 2010) to food odours. In comparison to the commonly used fly food containing a large amount of essential proteins the odourless medium has only a few trace elements (Tab. 1, Fig. 4B). Flies were transferred daily into fresh vials containing Thorpe´s medium.

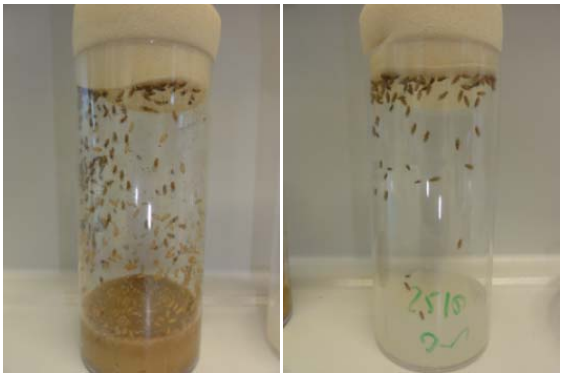

Figure 4: *D. melanogaster* was reared on special fly food medium.
Flies in a tube with conventional cornmeal medium **(A)** and an artificial, nearly odour-free medium (Thorpe's medium) **(B)**.

In order to investigate the function of AKH during starvation processes I used Canton-S wild type flies (WT) and two transgenic fly lines modified with the help of the GAL4/UAS system (see section 2.2). Both strains, *dAkh-gal4* needed for the specific expression and an UAS line with the corresponding gene *rpr* (Tab. 2), are available at the Bloomington *Drosophila* stock centre. By crossing UAS-*rpr* with a *dAkh-gal4* driver the programmed cell death of adipokinetic hormone positive cells in the first filial generation *(dAkh-gal4*/UAS-*rpr*=AKH^{-}) is initiated.

Table 2: List of used transgenic fly lines.
BL-Nr.=Bloomington stock number, CC=corpora cardiaca

Fly Line	BL-Nr.	Genotype	Source	Function
UAS-*reaper*	5824	w[1118]; P{w[+mC]=UAS-rpr.C}14	APLIN *et al.*, 1997	Initiation of programmed cell death (apoptosis),
dAkh-gal4	25683	y[1] w[*]; P{w[+mC]=Akh-gal4.L}2/ CyO, y[+]	LEE & PARK *et al.*, 2004	Expresses GAL4 in AKH-cells of the CC

For my experiments, female virgin flies of *dAkh-gal4* and males of the fly line UAS-*rpr* were collected. About twenty virgins and five males were put together into each vial. After 10 to 12 days the first daughter generation, called *dAkh-gal4*/UAS-*rpr* (1) eclosed. In order to ensure that the crossing yielded two functionally identical crossbreedings I also produced *dAkh-gal4*/UAS-*rpr* (2) in the same way. For this purpose female virgins of UAS-*rpr* and male flies of *dAkh-gal4* were grouped together. Figure 5 gives an

overview about the crossing of the required fly lines.

In order to exclude maternal effects pre-experiments were performed in a binary trap assay (see section 2.3.3). Both filial generations, *dAkh-gal4*/UAS-*rpr* (1) and *dAkh-gal4*/UAS-*rpr* (2), should be genetically identical in females. In order to examine, whether they are also similar in behavioural responses, I investigated ethyl acetate attraction in female flies. Both crossings showed similar olfactory responses when tested non-starved, and after one, four and 28 hours of starvation (data not shown). They were not distinguishable in their behavioural response to an odorant. Thus, *dAkh-gal4*/UAS-*rpr* (2) flies were used in further experiments.

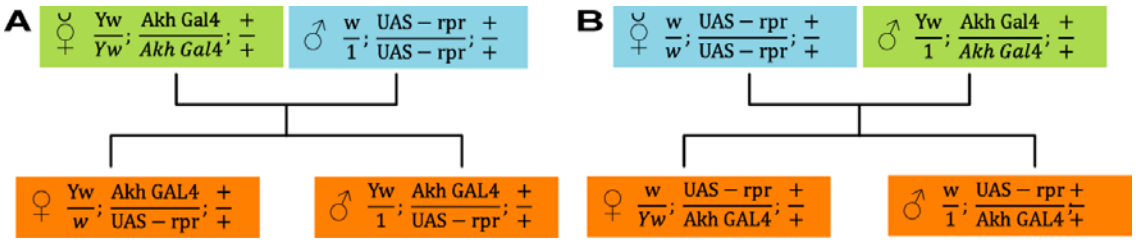

Figure 5: Model of crossbreeding.
Green: flies containing homozygous *dAkh-gal4*, blue: flies containing homozygous UAS-*rpr*, orange: generated heterozygous fly lines **(A)** *dAkh-gal4*/UAS-*rpr* (1) and **(B)** *dAkh-gal4*/UAS-*rpr* (2).

For this study experiments were performed in mated females (except for the mortality assay). Throughout the experiments I tested WT flies and in addition to that the genetically closer related parental generation as control groups to exclude the possibility that the genetic background influences starvation effects. Thus, four different groups of flies were arranged, that were starved for different time periods determined by the experiment. Depending on to the starvation time, before starting the experiments the flies were transferred into a new vial without food but containing a moist paper.

3.2 Chemicals

I mainly used odorants which are commonly used in studies with *D. melanogaster*. All odorants were commercially available in the highest degree of purity. Food-related odorants were used, since the response of female flies is higher than those of males

(STECK *et al.*, 2012). Ethyl acetate is a known attractive plant-odorant component (HUTNER *et al.*, 1937; VISSER, 1986). Two further odorants that were used in single sensillum recording (SSR) (2-heptanone, ethyl butyrate) only functioned as an indicator for the preferred neuron type of basiconic sensilla ab2 (DE BRYNE *et al.*, 2001). The set of odorants and the used concentrations are shown in Table 3.

Table 3: List of used odorants.
C= concentration, CHEM.= chemical, ETA= Ethyl acetate, ETB= Ethyl butyrate, HEN=2-Heptanone

Odorant	Chem. Formula	CAS-No.	Chem. Class	Company	$C_{t\text{-}maze}$	C_{SSR}
ETA	$C_4H_8O_2$	141-78-6	Ester	Aldrich	10^{-3}	10^{-2},10^{-3},10^{-4},10^{-5}, 10^{-6},10^{-7},10^{-8}
HEN	$C_7H_{14}O$	110-43-0	Ketone	FLUKA	-	10^{-3}
ETB	$C_6H_{12}O_2$	105-54-4	Ester	SIGMA	-	10^{-3}

3.3 Electrophysiology

3.3.1 Single Sensillum Recording – Odour Response of the Antenna

Animal Preparation

For measuring the physiological response of the *Drosophila* to an odorant at the first level of odour coding, extracellular electrophysiological recordings of OSNs, which are known to respond to the tested odorants, were performed on the antenna (single sensillum recording). For this purpose I used the transgenic flies *dAkh-gal4*/UAS-*rpr* (2) comparing them to WTs as well as to *dAkh-gal4* and UAS-*rpr* fly strains. Three starvation conditions were used: zero, four and 28 hours.

Single female flies of every genotype (see section 3.4.1) were blown into a pipette tip with only their head, especially the anterior half of the eyes and the antennae, protruding (Fig. 6). Flies were thus immobilized. The pipette tip was placed on an object slide covered with wax. Since ab2 sensilla are abundant on this side of the antenna (see section 2.3), the fly's ventral side tending upwards. Furthermore, the animal wax prevented the fly from backing out. Subsequently, the right antenna was fixed on a cover slip by a thin glass capillary between the second and third antennal segment.

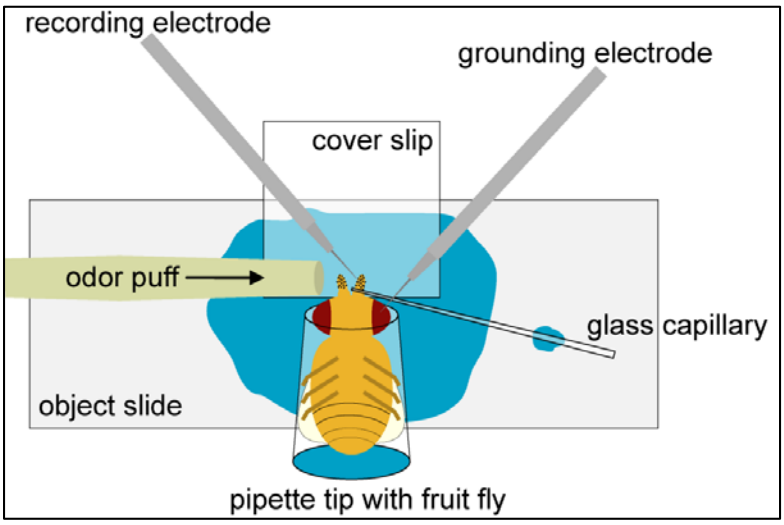

Figure 6: Measuring olfactory response via single sensillum recording. The female fly was blown in a pipette tip before positioning it on an object slide. The ventral side points upwards. A glass pipette helps to fix the antenna on the cover slip. The Position of the two electrodes is shown. Stimulation via odour puff is given through an odour delivery tube. Blue colour implies wax.

Recording the Olfactory Response

For extracellular recordings tungsten electrodes were used. Prior to the experiment they had to be electrolytically sharpened by immersing in a KNO_2 solution. After putting the object slide under the microscope the grounding electrode was positioned in the eye of the fly (Fig. 6). For measuring the olfactory response of the OSNs of interest the recording electrode was put into the large basiconic sensilla, which are primarily responding to food odours (DE BRYNE *et al.*, 2001; FISHILEVICH, E. & VOSSHALL, L. B., 2005).

Type ab2 sensilla were identified based on morphology (see section 2.3.) and on known responses to diagnostic odorants: 2-heptanone and ethyl butyrate (DE BRYNE *et al.*, 2001). For each starvation duration, odorant and concentration, one sensillum per fly was recorded (N=7). Changes in extracellular potentials were measured with the computer software Auto Spike32 (version 3.7). The stimulus controller Sytech IDAC-4 controls and defines the properties of the odour puff (10x probe, sampling rate 10,666 Hz). For my studies, the pulse duration was 500 milliseconds. The computer program recorded the response from two seconds before to ten seconds after pulse stimulation. A main flow and pulse flow of about 0.3 l/min was realized. In order to exclude mechanical artefacts, a compensatory flow was interposed when the fly was not stimulated.

The serial dilutions for the single sensillum recording (for concentrations see section 3.2.) were made with fluid paraffin oil. It was also used as a control. As a further control

stimulus I only took the blank filter paper to exclude contamination and to test the pure air flow. 10 µl of each dilution and control were dropped on a filter paper placed within a glass pipette which was used for stimulation.

3.4 Behavioural Measurements

3.4.1 The *Flywalk* – Measuring Fly Activity

I examined the activity pattern of different genotypes of vinegar flies by using the *Flywalk* (STECK *et al*., 2012). Four to five days old flies raised on cornmeal medium were transferred for one day into a vial containing a moist paper towel. Experiments were performed using the same genotypes as described above. Single flies were inserted into 15 parallel glass tubes which had a length of 18 cm and a diameter of 0.8 cm (Fig. 7).

During the measurement a laminar and steady air flow (0.3 l/min) was blown through the individual tubes. It was controlled by air regulators and measured by digital flow meters. Water bottles connected with the air flow ensured a humidity of about 70% and a surrounding temperature of 20°C. The humidity and the temperature were quantified in an additional glass tube without animals. Fly activity was recorded in darkness. An electroluminescent foil (from Reichel Elektronik, Germany) covered by a red filter foil increased the contrast for the camera. The filter foil transmitted light with wavelengths of >630 nm (maximum 640 nm). This was invisible to the flies so that external visual cues could be excluded (YAMAGUCHI *et al.*, 2010). Animals could move freely within the whole tube. Every ten minutes they were tracked for 20 seconds by an automated tracking algorithm. Two connected computer programs (LabVIEW 2009 and AnTS) controlled stimulation and tracking information. They were recorded for a maximum of two days. Measurements were performed always at the same time of day.

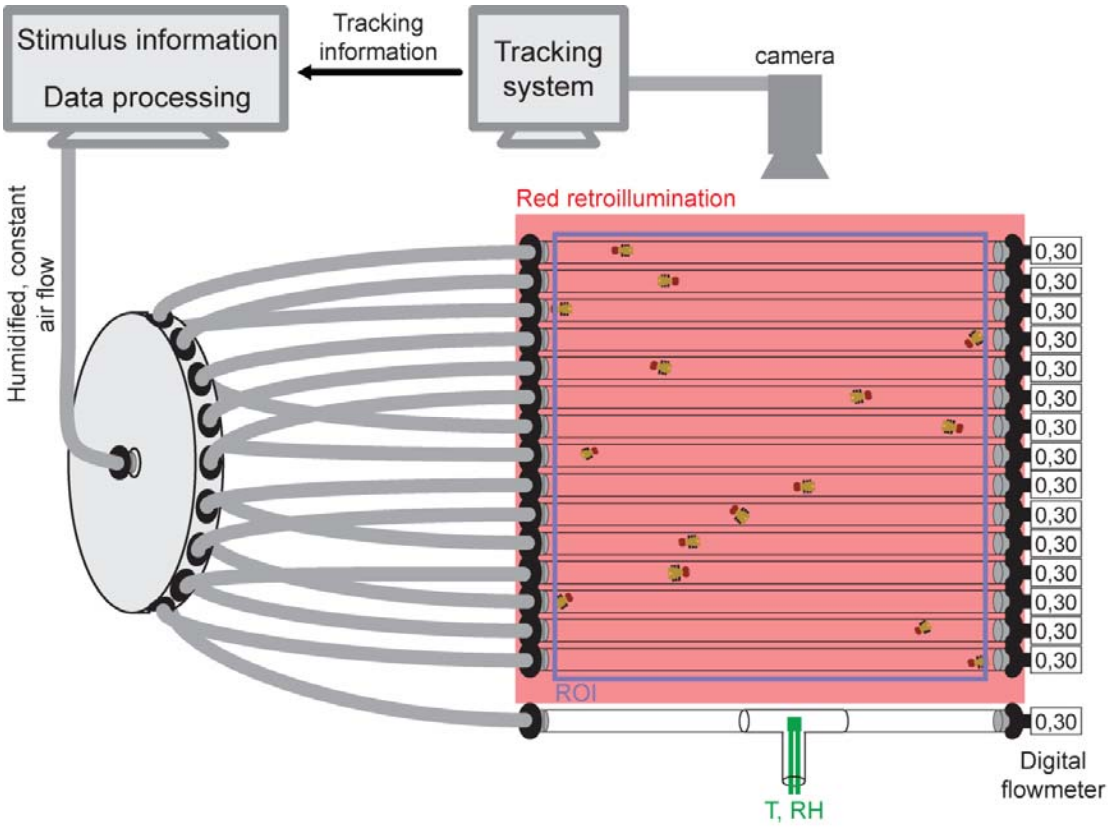

Figure 7: The *Flywalk* system - Tracking velocity (cm/s) of 15 separated flies.
Red retroillumination allowed recording in darkness. Stimulation and tracking information were controlled by two connected computer programs (LabVIEW 2009 and AnTS). Humidified air flow in individual glass tubes was controlled by air regulators and measured by digital flowmeters. Also, humidity and temperature (RH, T) were measured in an additional empty glass tube. Recordings were performed every ten minutes for 20 seconds. T=temperatur, RH=relative humidity, ROI=region of interest (modified by STECK *et al*., 2012).

3.4.2 Mortality Assay – Measuring the Survival Rate of *D. melanogaster*

In order to quantify the survival rate of *D. melanogaster* a starvation-induced mortality assay was implemented. For this purpose I used the same fly lines as already described (see section 3.1). About 50 eclosed animals (mated males and females in approximately equal numbers) of each genotype were transferred into a separate vial containing a moist paper. In order to synchronize starvation-induced death all genotypes were transferred simultaneously. The vials were placed in a climatic chamber (Snijders Scientific) with 25°C, 70% humidity and 12 hours day-night cycle. Experiments were performed always at the same time of day in order to exclude an impact of circadian rhythmic activity. The number of dead flies was counted after certain time intervals until the death of all flies (after 108 hours at the latest).

3.4.3 Binary Trap Assay (T-Maze) – Measuring Odour-Guided Behaviour

Preparation

With the following described techniques odour-guided behaviour in flies can be quantified. The behaviour of female fruit flies was measured in a trap assay termed t-maze (Fig. 8). 15 to 30 female flies were separated on ice, collected for starvation into small glass bottles containing humid cellulose papers, and afterwards transferred into the t-maze where the flies had to decide between two traps. An additional group of flies that was fed properly (zero hours of starvation) served as a control. They were transferred into bottles filled with the odour-free medium one hour before testing.

The binary trap assay was composed of a polypropylene t-connector (from Carl Roth GmbH&Co.KG article E768.1), two prepared microfuge tubes (1,5 ml) with integrated microtips as traps and a lid closing the third arm of the t-maze (Fig. 8A, B). Microtips were cut in a suitable size so that the flies could enter them. They were inserted into the sliced bottom of the microfuge tubes. Thus, flies were able to move into the trap but were not able to return (one-way trap door). Within the t-maze flies could then choose between a trap containing an odorant (odour trap) and a control trap without the odour (control). The odour trap contained the odorant (diluted in water) (for the concentrations see Chapter 3.2 Chemicals) which was heated with 1% low melting point agarose (LMA) and was placed into the cap of one microfuge tube (control trap). The control trap contained water with agarose but no odorant. The lid of the t-connector's third arm also contained water with 1% LMA. Five groups of flies were tested: *dAkh-gal4*/UAS-*rpr* (1), *dAkh-gal4*/UAS-*rpr* (2) as well as WT, *dAkh-gal4* and UAS-*rpr* as control groups.

Conditions and Measurement of Olfactory Response

In order to measure the olfactory-guided behaviour, six t-mazes per test chamber were placed on white filter paper. For the arrangement of the single t-mazes, see Figure 8C. On three sides, the assay was covered with black paper. Thereby visual cues influencing the response of *Drosophila* could be minimized. The observation could then be made through the fourth side that was open and accessible. Experiments were performed in climate chambers with an ambient temperature of 20 °C, a humidity level of 70 % and 12 hours day-night cycle. In each t-maze about 15 and 30 flies were

introduced carefully. Afterwards they were observed for 40 minutes. After particular time intervals (2,5; 5; 7,5; 10; 15; 20; 25; 30 and 40 minutes) The number of flies in the control trap as well as the odour trap and the residual t-connector were recorded after particular time intervals (2.5; 5; 7.5; 10; 15; 20; 25; 30 and 40 minutes). The response index (RI) was than calculated as the quantity of flies in the odour trap minus the quantity of flies in the control trap divided by the total number of flies (for further information see section 3.5).

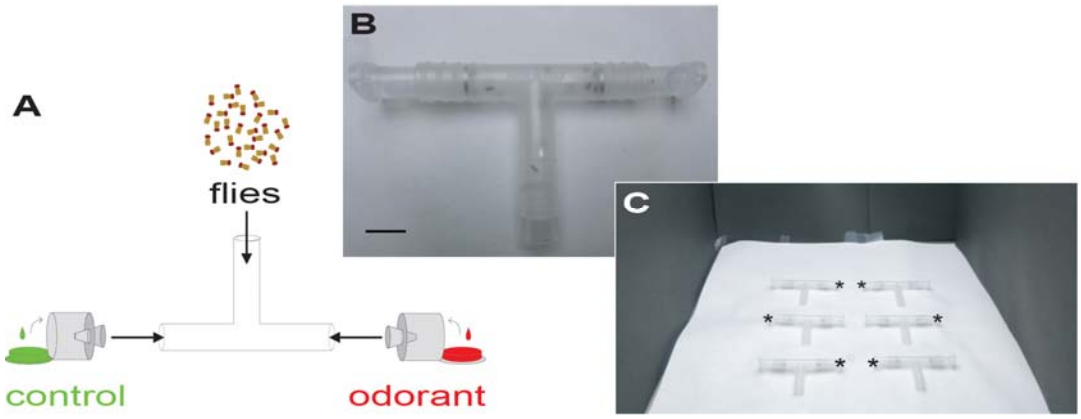

Figure 8: Measuring the olfactory response with a binary trap assay (t-maze).
(A) T-connector with two modified microfuge tubes acts as one-way trap door. Flies were transferred into the third arm. **(B)** Prepared t-maze after introducing the flies. Scale bar: 1 cm. **(C)** Arrangement of one experiment with six trap assays. Stars indicate the odour trap.

3.5 Data Analyses and Statistics

Single Sensillum Recording.

The computer software Auto Spike32 (version 3.7) detected changes in extracellular potentials defined as physiological response (spikes) of tested flies. By sorting spike amplitudes responses of ab2A neurons were filtered. The firing rate in response to odorant stimulation in ab2A neurons and the spontaneous activity were analysed. For investigating the frequency over the total recording interval, a bin width of 25 ms was used. However, for the evaluation of spontaneous activity patterns of firing neurons, I observed the mean frequency of the recording interval in which all tested flies were stimulated with paraffin oil or filter paper. In order to determine the response to the odorant, in all flies the maximum frequency during stimulation was quantified and

arithmetically averaged. For each concentration of ethyl acetate the maximum frequency during stimulation with paraffin oil was subtracted.

The Flywalk.

The computer programme MatLab interpolated a fly's velocity (cm/s) based on spatial and temporal recordings for intervals of 0.1 s. Since flies were recorded every 10 minutes for a total tracking interval of 20 seconds per fly and hour, a maximum of six single time traces with 200 single velocities each could be obtained. The percentage of the activity (velocity≠0) per single time trace was calculated. The median of maximal six activities (%) per fly and hour was quantified. Subsequently, the arithmetically average for all 30 flies per hour was calculated.

Mortality Assay

The survival rate for each experiment was calculated for the periods within the interval of 108 hours. Afterwards, the data were interpolated by R Studio (R 2.15.0) and additionally, the time was calculated when 50% of flies were still alive (half-life period).

Binary Trap Assay.

In order to calculate the response index RI (-1≤ RI ≤1, Fig. 9), the difference between the number of flies counted in the odour trap after 40 minutes and in the control trap was calculated and the result was divided by the total number of flies (see formula next page).

$$RI = \frac{Quantity\ of\ flies\ in\ the\ odour\ trap - Quantity\ of\ flies\ in\ the\ control\ trap}{Total\ number\ of\ flies}$$

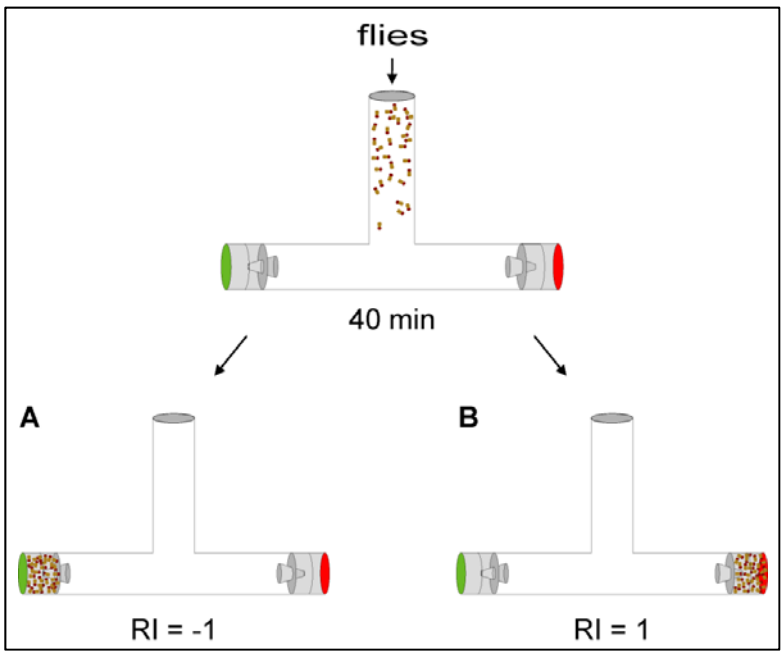

Figure 9: Measuring olfactory response with a binary trap assay – Calculating the response index.
After introduction, flies can move freely for 40 minutes within the t-maze. **(A and B)** Quantification afterwards shows the two possibilities of a maximal attraction index (AI). Red=odorant, green= control. **(A)** Flies are repelled by the odorant. All of them are counted in the control trap (RI=-1). **(B)** Flies were attracted by the odorant and are located in the odour trap (RI=1).

All obtained data sets were then analysed and processed with Microsoft Excel (version 2007). For statistical analyses InStat Software (GraphPad InStat 3) was used and either a Wilcoxon rank sum test, Mann-Whitney U test or a Kruskal-Wallis with Dunn´s Multiple Comparison post hoc test, were performed depending on the experiments. Graphics were plotted with SPSS Statistics 17.0 or R Studio (R 2.15.0). By means of Adobe Illustrator (11.0) all figures and graphs were edited.

4 Results

An animal which has been without food for a longer time may have a larger motivation to find energy resources than a fed one. Previous work with *D. melanogaster* showed a rise in glomerular activity to an odorant when the fly got hungry (ROOT *et al.*, 2011). Investigations on this topic are an important step to gain a deeper insight to understand altering and processing olfactory information by external conditions and the internal state.

4.1 Odour Response on the Antenna

Shortly after a fly has detected an odour, the olfactory neural circuit triggers a specific context-related behaviour. The odour signal has to be detected by the sensory neurons and has to pass through several regions of the insect's brain that transform, modify and filter olfactory information. Transforming chemical information of an odorant into an electrical signal within the olfactory sensory neurons is the initial step of olfactory processing of fruit flies. I wanted to investigate these effects at the level of the sensilla. In this context, I raised the following questions: Does starvation alter the response of the sensillum? If yes, does AKH have an impact on this process?

In order to test this, I performed SSRs on large basiconical sensilla (BS) and used ethyl acetate in various concentrations (decadic steps, see section 3.2). It was shown that particularly type II BS show a response to ethyl acetate (DE BRYNE *et al.*, 2001). For my experiments, I solely wanted to test the response of the ab2A neurons. To distinguish the spike pattern of an ab2 sensillum from an ab3, which are both large basiconical sensilla containing two neurons, two additional odorants were used (ethyl butyrate, 2-heptanone). ab2B and also ab3A neurons show an increase in spike frequency after stimulation with ethyl butyrate. If the sensillum is exposed to 2-heptanone I expected a high firing rate of ab3B, but none or just a low firing rate of ab2A (DE BRYNE *et al.*, 2001).

Initially, the spontaneous activity of AKH^{-} flies (N=7) and control animals (WT, UAS-*rpr*, *Akh-gal4,* N=7 per genotype) were analysed (Fig. 10). In WT flies spontaneous activity

increased with continuing starvation from 8.5±0.6 spikes/s (fed) to 11.3±0.4 spikes/s (28 hrs starvation), whereas AKH$^-$ flies display a rising firing rate after four hours of starvation. Subsequently, it decreased to the frequency similar to that of fed flies (Fig. 10). Thus, after longer starvation (28 hours) AKH$^-$ flies differed significantly from WT flies in spontaneous firing activities ($p<0.05$, $N_{AKH-}=15$, $N_{WT}=19$, Kruskal-Wallis, Dunn´s Multiple Comparison test).

When recording the genetic control, the frequency of both parental fly strains (UAS-*rpr*, *Akh-gal4*) remained constant before and after starvation, with UAS-*rpr* flies exhibiting a higher firing rate than *Akh-gal4* flies. This yielded in a spontaneous activity of AKH$^-$ flies intermediate between those of the mentioned strains (Fig. 10).

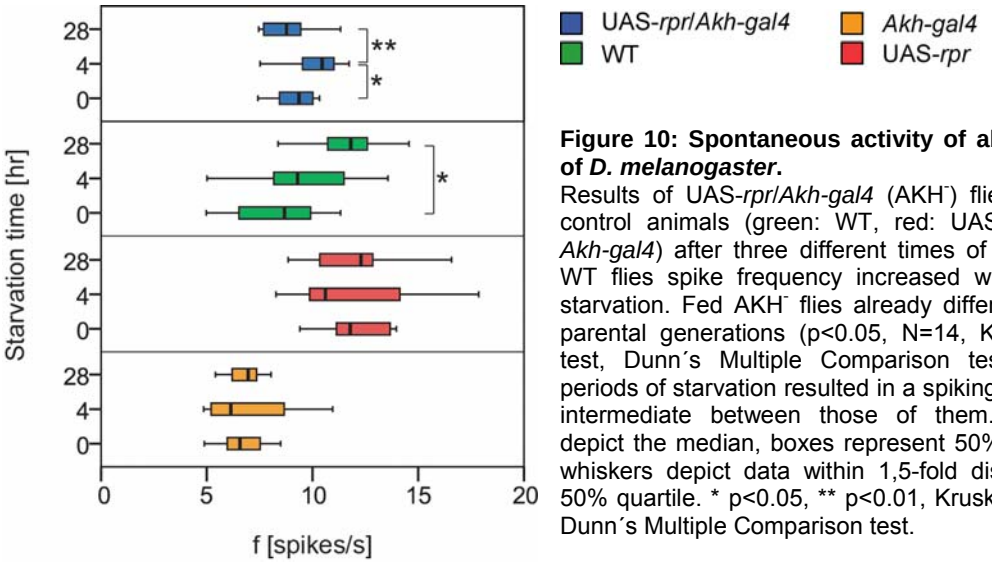

Figure 10: Spontaneous activity of ab2A neurons of *D. melanogaster*.
Results of UAS-*rpr*/*Akh-gal4* (AKH$^-$) flies (blue) and control animals (green: WT, red: UAS-*rpr*, orange: *Akh-gal4*) after three different times of starvation. In WT flies spike frequency increased with continuing starvation. Fed AKH$^-$ flies already differed from their parental generations ($p<0.05$, N=14, Kruskal-Wallis-test, Dunn´s Multiple Comparison test). All three periods of starvation resulted in a spiking frequency (f) intermediate between those of them. Black lines depict the median, boxes represent 50% quartile and whiskers depict data within 1,5-fold distance of the 50% quartile. * $p<0.05$, ** $p<0.01$, Kruskal-Wallis-test, Dunn´s Multiple Comparison test.

The data showed that starvation up to 28 hours is associated with a rise in spontaneous firing rate of ab2A neurons in WT flies. This starvation-induced increase had not been found in AKH$^-$ flies after longer starvation (28 hours). However, the obtained results suggest that the finding of AKH$^-$ flies might depend on the genetic background and no conclusion could be drawn about the relation between firing pattern and duration of starvation.

I next analysed the response of ab2A neurons to ethyl acetate. The difference between the maximum frequency during odorant and paraffin oil stimulation per second were calculated (=Δspikes/s). The results yielded a dose-dependent firing (Fig. 11) in all genotypes.

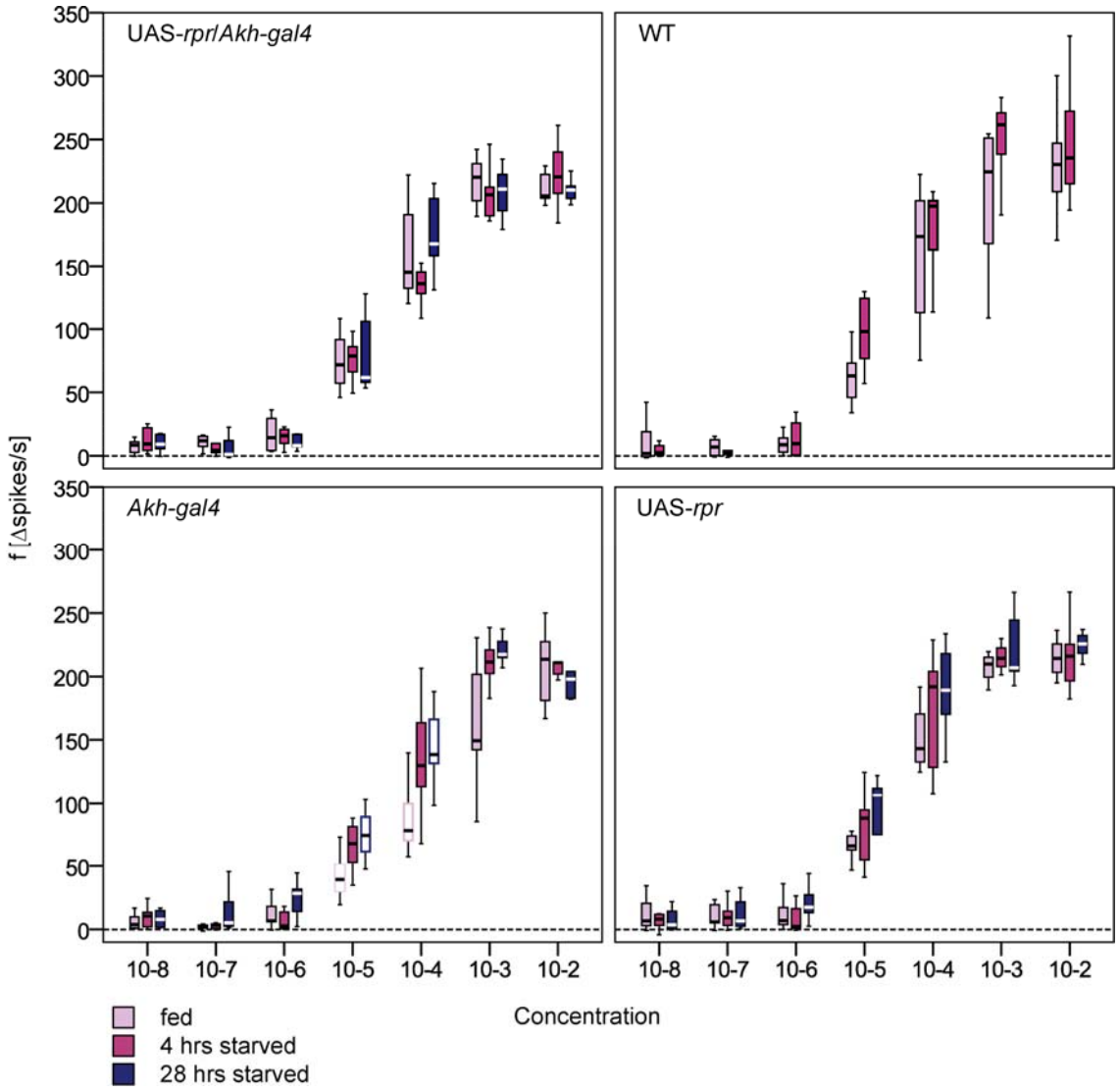

Figure 11: Response of ab2A neurons to ethyl acetate stimulation.
The maximum firing rate during 500 ms of stimulation is plotted against the concentrations of ethyl acetate (Δspikes=maximum frequency during stimulation with the odorant – maximum frequency during paraffin oil stimulation). Results of UAS-*rpr*/*Akh-gal4* (AKH^-) flies and control animals (WT, UAS-*rpr*, *Akh-gal4*) after three different times of starvation (light pink: fed, dark pink: 4 hrs starvation, blue: 28 hrs starvation). In all genotypes doses-dependent spike frequencies had been recorded. 28 hours starved WT flies were tested with another set up. Obtained data were not comparable and were not shown in the graph. Empty boxes indicate significant differences ($p<0.5$, Kruskal-Wallis, Dunn´s Multiple Comparison test). Black lines depict the median, boxes represent 50% quartile and whiskers represent data within 1,5-fold distance of the 50% quartile.

Although no significant differences were obtained, starved WT flies seemed to respond with higher spike frequency by stimulating the insect with concentrations of ethyl acetate between 10^{-3} and 10^{-5} (dynamic range of ab2A neurons for ethyl acetate) (Fig. 11). 28 hours starved WT flies had to be measured with a second set up. Thus, obtained data were considered to be too different and were not taken into account here. Contrary to WT flies, after stimulation, AKH^{-} flies exhibited similar frequency patterns not depending on their starvation time (Fig. 11).

When testing the parental fly strains, a continued starvation for up to 28 hours increased the spike frequency of ab2A neurons in *Akh-gal4* flies significantly after they had been stimulated with concentrations between 10^{-4} and 10^{-5} (Fig. 11). A similar tendency was noticeable in UAS-*rpr* flies within the dynamic range, although no significant differences could be found. In *Akh-gal4* and UAS-*rpr* flies the biggest increase in spontaneous activity is observed after the first four hours of starvation and afterwards the spike frequency tended to remain constant after extended starvation. The results of the SSR experiments indicate that there might be an influence of AKH on starvation effects. A considerable tendency in increased spike response had been observed in control animals. In *Akh-gal4* flies the tendency was significant.

4.2 Odour-Guided Behaviour – Response to an Attractive Odorant

Having examined the physiological effects of starvation, I now wanted to investigate these effects in behaviour as a result of information transmission and processing in olfactory circuits. How does the duration of starvation influence the attractiveness of an odorant? Investigations of ROOT *et al.* (2011) and FARHAN *et al.* (in preparation) showed an increase in odour-mediated responses in starved fruit flies. In order to verify these findings, I tested flies in a binary trap assay, the t-maze. By repeating the tests with WT flies, I confirmed the published findings of increased attractiveness after starvation (Fig. 12B). Flies, that had not been starved, did not show any preference for ethyl acetate (10^{-3}) or the control (Fig. 12). After one hour much more flies could be counted in the odour trap. In the first four hours without food ethyl acetate became rapidly attractive. By prolonged starvation up to 28 hours the mean RI remained stable at 0.57±0.07.

Recent studies have already investigated starvation effects of the AKH (LEE & PARK, 2004). However, the impact of starvation on olfactory perception related to AKH was not examined. In order to test the hypothesis, that AKH influences the attractiveness of an odorant in hungry vinegar flies, AKH$^-$ flies were tested in the same assay (Fig. 12). This yielded a rise of attraction of ethyl acetate up to 0.22±0.09 in the first four hours. AKH$^-$ flies only exhibited high preferences to an odorant after a starvation of 28 hours.

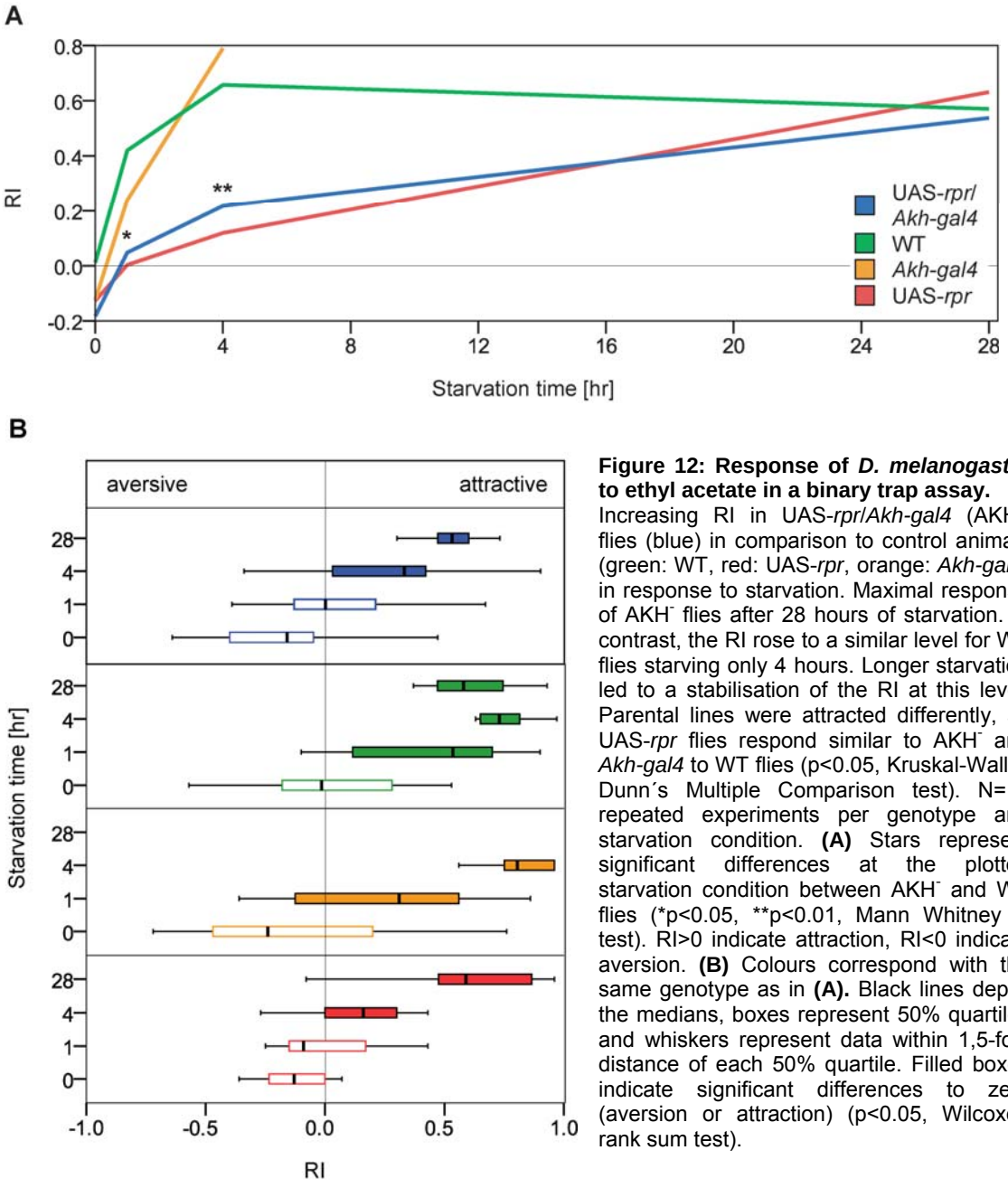

Figure 12: Response of *D. melanogaster* to ethyl acetate in a binary trap assay. Increasing RI in UAS-*rpr*/*Akh-gal4* (AKH$^-$) flies (blue) in comparison to control animals (green: WT, red: UAS-*rpr*, orange: *Akh-gal4*) in response to starvation. Maximal response of AKH$^-$ flies after 28 hours of starvation. In contrast, the RI rose to a similar level for WT flies starving only 4 hours. Longer starvation led to a stabilisation of the RI at this level. Parental lines were attracted differently, as UAS-*rpr* flies respond similar to AKH$^-$ and *Akh-gal4* to WT flies ($p<0.05$, Kruskal-Wallis, Dunn´s Multiple Comparison test). N=12 repeated experiments per genotype and starvation condition. **(A)** Stars represent significant differences at the plotted starvation condition between AKH$^-$ and WT flies (*$p<0.05$, **$p<0.01$, Mann Whitney U test). RI>0 indicate attraction, RI<0 indicate aversion. **(B)** Colours correspond with the same genotype as in **(A).** Black lines depict the medians, boxes represent 50% quartiles and whiskers represent data within 1,5-fold distance of each 50% quartile. Filled boxes indicate significant differences to zero (aversion or attraction) ($p<0.05$, Wilcoxon rank sum test).

Responses in fed flies did not differ between WT and *Akh-gal4*/UAS-*rpr* (Fig. 12). Both genotypes were either attracted to ethyl acetate or repelled. AKH^- flies were significantly less attracted than WT within the first four hours of starvation (Fig. 12A). However, after starving up to 28 hours the odorant became highly attractive to AKH^- flies, as I could demonstrate in WT flies before. In both genotypes the response to ethyl acetate increased with continuing starvation. In contrast to AKH^- flies, the RI in WT already stabilised after four hours with only water.

The results showed that flies without AKH differed from the wild type in their behavioural response to an attractive food-related odorant in the first four hours of starvation. After a very long starvation time the effect vanished.

In order to exclude the possibility, that the genetic background caused the effect instead of the targeted AKH-cell apoptosis, I also tested the parental fly lines, which were related closest, in the binary trap assay. In Figure 12 the results of all genotypes and starvation conditions are shown. *Akh-gal4* flies have not been tested after 28 hours of starvation, since they had died too quickly (see section) resulting in too few animals for the experiments.

The attractiveness of ethyl acetate was different in the transgenic parental generations. *Akh-gal4* and WT flies exhibited a similar behaviour to ethyl acetate, whereas the parental fly strain UAS-*rpr* responded as I could already demonstrate in AKH^- flies (Fig. 12A). AKH^- and UAS-*rpr* flies only reached a maximum after 28 hours of starvation. On the contrary, in WT and *Akh-gal4* flies starving up to four hours already increased the response to ethyl acetate to a maximum. The response to the odorant related to starvation was already different in the genetic controls, of which one yielded similar results to AKH^- flies. Thus, the influence of AKH on starvation-increased attractiveness of odorants remains unclear.

4.3 Verifying Starvation-Induced Survival Rates and Activity Patterns

Starved flies have to use their stored nutrients to survive until finding new energy sources. In order to increase the chance of detecting food, starved flies become hyperactive showing intense food search behaviour prior to death (LEE & PARK, 2004). In contrast, in AKH$^-$ flies hyperactive behaviour is suppressed.

I investigated this starvation-mediated behaviour while measuring the activity of *D. melanogaster* in the *Flywalk*. When testing AKH$^-$ and WT flies, I confirmed this finding, as the WT exhibited an increased activity of about 20%, which lasted for about 10 hours before they died, AKH$^-$ flies did not. When testing parental lines in the same assay, I observed increased activation in both lines prior to death (Fig. 13) suggesting, that the finding of AKH$^-$ flies did not just depend on the genetic background.

My data confirm the investigations of LEE & PARK (2004). Starved AKH$^-$ flies did not exhibit an enhanced pre-death activation. Furthermore, the initiation of starvation-induced mortality started later.

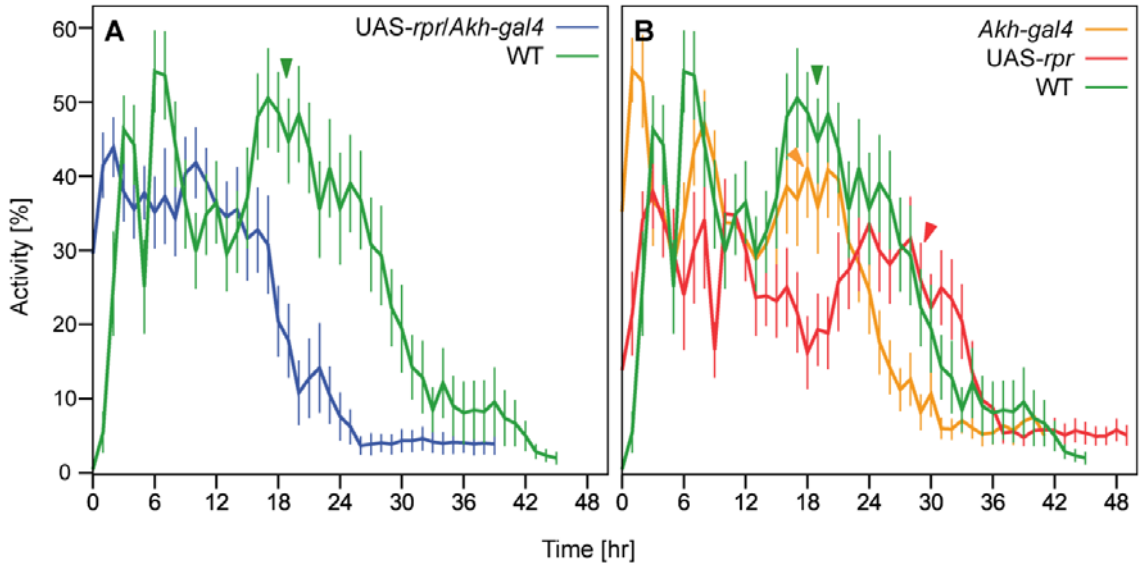

Figure 13: Activity pattern of different genotypes of *D. melanogaster* upon starvation.
(A) Contrary to WT flies (green) UAS-*rpr*/*Akh-gal4* (AKH$^-$) flies (blue) exhibited no rise in activity prior to death as well as weaker rhythmic activity. **(B)** This starvation-mediated effect observed in WT was also obtained in the genetic control (red: UAS-*rpr*, orange: *Akh-gal4*). **(A and B)** Irregular rhythmic activity patterns could be observed in all tested genotypes. In control groups the demonstrated hyperactivity (arrow heads) lasted about 10 hours. N=30 tested flies per genotype. Bars represent SEM.

It has already been shown that AKH$^-$ flies survive longer than control flies when food is not available (LEE & PARK, 2004). These results could be found regardless of gender. My experiments in the *Flywalk* yielded WT flies survived longer than AKH$^-$ flies. The results did not correspond with recent studies of LEE & PARK (2004). Therefore, I used an already published experimental design. To investigate the findings of LEE & PARK (2004) the mortalities of UAS-*rpr*/*Akh-gal4* and control flies (WT, UAS-*rpr*, *Akh-gal4*) were investigated by use of mortality assays (LEE & PARK, 2004).

I placed about 60 flies of each genotype into fly tubes and counted the flies until all animals were dead. With continuous starvation the survival rate of the genotypes decreased. Even though parental lines displayed a lower survival rate than the WT, it still increased compared to the WT in AKH$^-$ flies (Fig. 14). After 26 hours the first WT flies died, the first AKH$^-$ fly, however, after 32 hours at the earliest. Contrary to the WT, 48 hours after the beginning of starvation significantly more AKH$^-$ flies survived (Fig. 14). 50 % of AKH$^-$ flies survived more than 70 hours. The half-life period of WT flies was calculated to 57.07±2.75 hours (mean±SEM). Within this time about 70% UAS-*rpr* and nearly all *Akh-gal4* flies (95%) died. The genetic control lived even shorter than the WT and the AKH$^-$ flies hence suggesting that AKH$^-$ flies were more resistant to starvation-induced death.

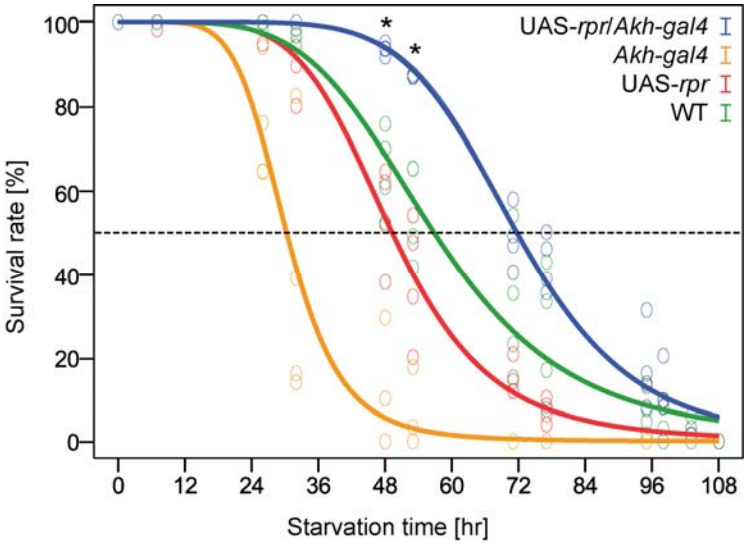

Figure 14: Survival rate of different genotypes of *D. melanogaster* upon starvation. When flies were starved with free access to water after 72 hours only 50% of UAS-*rpr*/*Akh-gal4* (AKH$^-$) flies (blue, n=249) died and between 68% and 100% of control animals (green: WT, n=245, red: UAS-*rpr*, n=230 orange: *Akh-gal4*, n=239). Circles indicate survival rate of 1 tube with ~60 flies. Bold lines represent fitted curves of means of 4 repeated experiments. Stars represent significant differences at the plotted point in time between AKH$^-$ and WT flies ($p<0.05$, Mann Whitney U test).

To summarize, the responding ab2A neurons of AKH- flies did not show any change after starvation in comparison to control animals, which tended to exhibit increased firing frequencies within the dynamic range of odorant stimulation. Despite this tendency, no significant differences had been found. My data also suggest that starved AKH^- flies were highly attracted to a food-related odorant only after extended starvation times and differ from WT flies in the first few hours of starvation. Results of the genetic controls yielded differences between the two transgenic parental lines. Therefore, differences between WT and AKH^- flies might depend on the genetic background. Furthermore, I confirmed the investigations of LEE & PARK (2004) about longevity and activity patterns. I could not exclude that starvation has had an influence on the behavioural and physiological responses of AKH^- flies or whether responses depend on the genetic background.

5 Discussion

Hunger is an internal physiological state that drives vertebrates and invertebrates to seek and ingest energy resources essential for their survival. With the support of the olfactory sense, insects are able to localize, evaluate and detect food sources. In this thesis starvation effects and a potential neuropeptide involved were investigated.

5.1 Starvation effect in WT flies

Drosophila feeds on microorganisms growing on decaying fruit (BECHER *et al.*, 2012, LACHAISE & TSACAS, 1983). Odours which can be connected either directly to target microorganisms or to inhabited fruits usually are attractive to flies (STENSMYR *et al.*, 2003; KNADEN *et al.*, 2012). For my studies, I used ethyl acetate. This food-related odorant is very attractive to *Drosophila* (HUTNER *et al.*, 1937; VISSER, 1986) and is encountered in their breeding-sites (JAENIKE, 1982). My behavioural and physiological investigations in *Drosophila* indicated an alteration of odorant attractiveness under starvation.

Odour-guided behaviour is the result of a complex neural circuit, which is influenced and altered by many external and internal factors in several levels of odour coding. Initially, odour molecules enter the olfactory appendages and sensilla of the insect, respectively. Since in *D. melanogaster* ethyl acetate is an attractive food-related odorant and the large type 2 basiconic sensilla (BS) respond strongly to the odorant (DE BRYNE *et al.*, 2001), I concentrated on ab2A neurons and analysed the spontaneous activity pattern of firing neurons as well as the maximum spike frequency in response to odorant stimulation in starved and fled flies. In a previous study ROOT *et al.*, (2011) observed starvation effects in the antennal lobe, especially an enhanced activity in DM1, DM2 and DM4 glomeruli. ab2A neurons, that I recorded in my study, project to the DM4 glomerulus (LAISSUE & VOSSHALL, 2008), so that my data could be directly compared with those of ROOT *et al.,* (2011). Although both studies used different odorants (ROOT *et al.*, 2011: cider vinegar), I already found a tendency in the peripheral organisation in ab2A neurons of four hours starved WT flies that were

responding with higher spike frequencies to ethyl acetate than fed animals. Additionally, the spontaneous activity of ab2A neurons increased with continuing starvation.

The tendentially increased physiological activity mentioned above was accompanied by an increased behavioural response. WT flies tested in a binary trap assay were highly attracted to the presented odorant already after 4 hours of starvation. Without any starvation no preference for the odorant stimulus was observed.

This suggests an impact of starvation, on the one hand, on the response of ab2A neurons particularly in the first few hours as it was already demonstrated by ROOT *et al.* (2011) in the antennal lobe. On the other hand my findings suggest an impact of starvation on odorant-guided, behavioural responses. AYYUB *et al.* (1990) did also perform experiments with binary trap assays. In comparison, starved *Drosophila* was similarly attracted by equal concentrations of ethyl acetate. Insects still require energy in the absence of available food and have to utilize stored substrates. This can be critical for their survival. Since they have to acquire new sources immediately, hungry flies may be more motivated to search for food. Therefore, food-related odorants gain higher relevance in starved than in fed flies. Furthermore, ROOT *et al.* (2011) found that an upregulation of activity in involved glomeruli (DM1, DM2, DM4) leads to more attraction to food-related odorants during starvation and enhanced food-search activities. The theory that hunger modifies behaviour is additionally supported by evidence of FARHAN *et al.* (in preparation), who also tested vinegar flies in a binary trap assay and found increased attraction to the odorant upon starvation. Additionally, they demonstrated that hunger modulates the response of OSNs in *D. melanogaster* while measuring faster spiking ab2A neurons in response to ethyl acetate. This was investigated even under 28 hours of starvation. In my study, recordings of 28 hours starved WT flies had to be conducted by a second SSR setup. It may have had differences in recording dependent on the measuring equipment. Odour contaminations of the second setup may be a possible reason for the differences in the results of this work. As the results from both setups deviated substantially, I excluded the data of the 28 hours starved WT flies. However, my data confirm this starvation effect in behaviour and indicate a similar tendency already in the periphery when exposed for four hours only with water.

Which neural circuits alter the odorant-mediated behavioural response of starved flies? Starvation-induced peripheral modulations may influence the central processing of olfactory information. Studies on *Drosophila* mutants, called *gigas,* have shown that an increase in the number of synapses of OSNs leads to higher olfactory responses (ACEBES & FERRÚS, 2001). After four hours of starvation there may be a reinforcement of activity in involved glomeruli. Longer starvation might lead to a generation of involved synapses as well as to abundant branching of OSNs as it was demonstrated in the *giga* mutants. I did not follow up this idea but raised the question, whether AKH is involved in the starvation effect.

5.2 The impact of AKH on the starvation effect

AKH is known to act as functional homologs of the vertebrate glucagon, which regulate blood sugar levels (NOYES *et al.*, 1995; VAN DER HORST *et al.*, 2001). Research of LEE & PARK (2004) underlines the role of AKH in starvation processes by testing hungry AKH^- flies. I wanted to investigate the influence of AKH on starvation effects regarding olfaction. Therefore, I created a fly line, which possessed no AKH, and expected that they did not exhibit starvation effects in olfactory perception as I showed in WT flies.

As discussed above, in WT flies I found a starvation-induced increase of spontaneous activity in the ab2A neurons as well as a tendency of increased firing rates in response to ethyl acetate. This shift had been observed especially during stimulation with ethyl acetate concentrations between 10^{-3} and 10^{-5} (=dynamic range of ethyl acetate in ab2A neurons). I therefore investigated whether this peripheral starvation effect depended on the presence of AKH. When recording ab2A neurons in AKH^- flies, I observed rising spontaneous activity within the first four hours of starvation. After extended starvation the firing rate regressed to the level of that of fed AKH^- flies and differed from spontaneous activity of 28 hours starved WT flies. Contrary to WT flies, animals without AKH did respond to ethyl acetate with similar firing pattern independent of starvation. The results imply an impact of AKH in starvation effects. When testing the parental fly strains, a constantly higher (UAS-*rpr*) and lower (*Akh-gal4*) level of spontaneous activities were recorded than in AKH^- flies. These differences in genetic control lines indicate that the genetic background may lead to differences in the firing pattern

between AKH^- and WT flies. Therefore, I am not able to draw a conclusion about the starvation effects in spontaneous activity of AKH^- flies. Recordings of genetic control lines after odorant stimulation yielded higher maximum frequency within the dynamic range of ethyl acetate in ab2A neurons in starved *Akh-gal4* flies. I could also demonstrate the same tendency in UAS-*rpr* flies. Thus, both parental fly lines responded like WT flies. The used sample size was only seven sensilla of seven different flies per genotype. Increasing the sample size might significantly confirm the tendency in higher responses between fed and starved control animals.

I next investigated, whether the absence of AKH affects the starvation-induced increase of behavioural response in flies. By testing AKH^- flies in the t-maze, I also observed a starvation-induced increase in the response to ethyl acetate. Contrary to WT flies and according to my investigations, AKH^- flies were highly attracted after 28 hours of starvation at the earliest. Since their response differed from that of WT flies in the first four hours when supplied only with water, AKH seems to influence early starvation effects. To test whether the effect did not derive from the parental line, I also investigated their responses. Since UAS-*rpr* flies were similarly attracted by the odorant as AKH^- flies and hence, differed from the *Akh-gal4* line, I cannot draw any conclusion about the impact of AKH on starvation effects.

Although I could not conclude whether the absence of AKH circumvents any starvation effect, I tested the AKH^- flies under another aspect. Shortly before WT flies die of hunger, they exhibit a drastic increase of activity that is discussed as a strategy to leave poor habitats and taking the risk of finding a new habitat (LEE & Park 2004). I therefore made use of the *Flywalk* paradigm to test whether the AKH^- flies that I used for my starvation experiments behaved like those published by LEE & Park (2004). By doing so, I could confirm these investigations about activity patterns under starvation conditions. AKH^- flies tested by the *Flywalk* exhibited an almost constant activity pattern upon starvation, whereas control groups became more active prior to death. Furthermore, LEE & Park (2004) observed diurnal, rhythmic activity patterns in fed *D. melanogaster* contrary to starved animals including WT, AKH^- and parental flies. As flies cannot feed during the *Flywalk* experiment, I could not investigate the activity patterns of fed animals. Consequently, only hungry flies can be compared with current researches.

As discussed above, activity before dying of hunger may reflect intense food-search behaviour as it has been established by ROOT *et al.* (2011). High activity needs much energy. If AKH$^-$ flies do not show hyperactivity prior to death, they had used less energy in contrast to their control animals. Hence, I expected prolonged survival in AKH$^-$ flies. A recent study of LEE & PARK (2004) showed that AKH$^-$ flies develop normally under usual growth conditions and do not exhibit defects in growth, metamorphosis, reproduction and courtship behaviour. However, under physiological stress (starvation) AKH$^-$ flies exhibit an extended longevity contrary to starved control flies. I did not observe this longevity effect in the *Flywalk*. Therefore, I used an already published experimental method, a mortality assay (LEE & PARK, 2004), in order to measure survival rates in *Drosophila*. AKH$^-$ flies exhibited resistance to starvation-induced death in comparison to WT flies even though their parental fly line died earlier than the WT.

To summarize, I expected that the lack of AKH in AKH$^-$ flies would have circumvented starvation-induced effects on the antenna as well as on their behaviour when comparing these flies with *Drosophila* WTs. However, I cannot draw any conclusion about the involvement of AKH in starvation effects of olfactory perception, since the data of the genetic controls varied extremely. The filial generations *Akh-gal4*/UAS-*rpr* (1) and *Akh-gal4*/UAS-*rpr* (2) did not differ in their behavioural response to ethyl acetate indicating that no maternal effects had been involved. In activity patterns and extended longevity AKH$^-$ flies behaved like it was already shown before.

However, due to not confirmed significant differences and unexpected results from the parental lines, it was not recognisable whether or not AKH is involved in starvation effects on olfactory perception. Consequently starvation might not influence odour-guided responses of AKH$^-$ flies, but in my experiments I could not completely answer this.

6 Outlook

Building on my experiments there are still issues for further investigations. Since I would expect that starvation engenders also an increase in sensitivity, future binary trap assays could be conducted using a wider range of concentrations of ethyl acetate in order to receive a doses-response curve of fed and starved flies. When examining sensitivity effects, particularly the responses to concentrations near the threshold, where fed flies just do not respond behaviourally, should be observed. After investigating a starvation-induced sensitivity effect, I would examine, whether the effects observed in my study are only limited for food-related odours or whether there are global effects and starvation has also influence on coeloconic and trichoid sensilla. In future experiments, a more complex odour set containing not only attractive food odorants, but also odorants like benzaldehyde, cis-vaccenyl acetate or ammonia could be tested physiologically and in behaviour.

This work includes peripheral effects and the resulting behaviour. Generated data of this study revealed that an attractive odorant becomes more attractive for *D. melanogaster* under starvation. Thus, further investigations could examine how the central olfactory processing in the antennal lobe is influenced by starvation. This could be done by optical imaging. On the one hand I would expect a starvation-induced reinforcement of glomerular input and a higher activity pattern of involved glomeruli. There is already evidence that the information for odorant relevance (attraction and aversion) is processed in certain glomeruli (KNADEN *et al.,* 2012). On the other hand a complete shift in glomerular response pattern might be observed.

The occurred differences between WT and AKH^- flies could result from a generally affected olfactory system in the transgenic fly line leading flies not to smell. In order to draw conclusions about this, the doses-response of fed WT flies should be compared with that of fed transgenic flies. I would expect similar doses-dependent responses, if the olfactory system is generally intact. The impact of the genetic background may result in a defective olfactory system. Furthermore, single sensillum recordings of AKH^- flies led to difficulties in the analyses of the results in contrast to control groups. Several tested morphological, large BS (concerning 30% of AKH^- flies) did neither

exhibit spontaneous activity nor show responses to the presented odorants (ethyl acetate, 2-heptanone, ethyl butyrate), although the electrode was brought into ab1, ab2 or ab3 sensilla. I could only record noise. Thus, solely flies showing action potentials were recorded. Research of THUM *et al.* (2006) showed that success in targeted apoptosis varies between cell types and that depends on the used method. It may be that AKH^- flies do not exhibit activity in neurons of BS whereas in transgenic flies *rpr*-induced apoptosis was not successful and they hence responded normally. Concerning the fact that AKH^- flies exhibited no spontaneous activity, a question needs to be answered is: Is there any activity pattern of the antenna or any responding neuron? By inserting a green fluorescent protein (GFP) into the *Akh-gal4* construct it can be visualized and proved if the *rpr*-induced apoptosis was successful or whether AKH-secreting cells can be detected within the insect brain. I would expect green fluorescence in the region of the corpora cardiaca by examining the parental *Akh-gal4* flies under a fluorescent microscope in comparison to their AKH^- filial generation expressing no GFP. Since parental fly lines differed from each other, when testing flies in a behavioural assay, it would be interesting to answer the question, whether other transgenic flies with a different genetic background but similar effects also show different responses in behaviour as well as in physiology. One possibility would be to exchange the UAS-*rpr* fly line. The apoptosis triggering gene *rpr* may have been partially ineffective (THUM *et al.,* 2006). The AKH-cell ablation could thus be realized using a different effector gene, for example the *Diphtheria toxin A*, which inhibits protein synthesis (HAN *et al*., 2000). The second possibility would be associated with the parental *Akh-gal4* line, which was balanced over *CurlyO* (*CyO*) on the second chromosome. I had expected that *CyO* will be out-crossed over several generations due to selection disadvantage. Surprisingly, the abundance of flies exhibiting a *CyO* phenotype remained constant in the *Akh-gal4* line. Although the fly strain was already tested and published (LEE & PARK, 2004), the described observation may indicate that the *Akh-gal4* insertion itself might influence the behaviour, possibly through positional effects of the P-element insertion. Therefore, I suggest to use another *Akh-gal4* construct like y[1]w[*]; P{w[+mC]=Akh-gal4.L}3 (see Bloomington Stock Center), in which Akh-Gal4 is inserted in a different position on the 3rd chromosome. Alternatively to the positional effects, different behavioural phenotypes may also be caused by the *yellow* mutation on the first chromosome, which has been reported to affect locomotor

activity by WILSON *et al.* (1976). This problem can be solved by crossing out the *yellow* mutation in parental fly lines.

Furthermore, LEE & Park (2004) observed diurnal, rhythmic activity patterns in fed *D. melanogaster* contrary to starved animals. As flies cannot feed during the *Flywalk* experiment, I could not investigate the activity patterns of fed animals. In order to exclude the possibility that pre-death enhanced activity could be the result of rhythmic activity pattern, fed flies should be tested in the *Flywalk* by placing a piece of sugar in each tube. Afterwards their activity pattern could be compared with starved animals and with current researches. Additional investigations should consider the use of Thorpe´s medium. During this work, flies were reared on Thorpe´s medium for a longer time and afterwards they were additionally starved. They generally seemed to be weaker which might be due to problems occurring when transferring flies on Thorpe´s medium. The lack of proteins in this medium may cause animals not to form particular amino acids. I cannot assess which implications this had on the response of the animals. IYENGAR *et al.* (2010) showed that flies are gaining olfactory experience on cornmeal medium that is different to that of flies, which are grown on artificial odourless medium. However, I am not aware of experiments that have been performed on lifespan or longevity on the nearly odour-free Thorpe´s medium. Does the use of the medium lead to disadvantages associated with this method? How is the longevity of flies influenced by growing on an artificial food medium? What differences would be observed when measuring starved flies growing on common cornmeal medium via SSR and in the binary trap assay? It would be interesting to investigate all of these aspects mentioned above.

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9 Declaration of Original Authorship

I hereby certify that this work presented is entirely my own and the result of my investigation. The material from other sources has been fully and properly acknowledged.

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