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The effect of larval olfactory experience on the olfactory sense of adult *Drosophila melanogaster*

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List of Abbreviations

AL	Antennal lobe
AP	Action potential
BEA	Benzaldehyde
ButA	Butyl acetate
cDNA	complementary DNA
Ctrl	Control
D	Dark
ddH_2O	Double distilled H_2O
ETA	Ethyl acetate
ETB	Ethyl butyrate
GFP	Green fluorescent protein
GPCR	G protein – coupled receptor
HexA	Hexanoic acid
KC	Kenyon cell
L	Light
LED	Light-emitting diode
LH	Lateral horn
LIM	S-(-)-limonene
IN	Local interneuron
IR	Ionotropic receptor
MB	Mushroom body
MOL	Mineral oil

N	Sample size: Quantity of tested flies
OBP	Odorant binding protein
OR	Olfactory receptor
Orco	Olfactory receptor co-receptor
ORN	Olfactory receptor neuron
PEEK	Polyetheretherketone
PN	Projection neuron
qPCR	Quantitative real-time polymerase chain reaction
ROI	Region of interest
RT	Room temperature
STDEV	Standard deviation
Tm	Melting temperature
UAS	Upstream activating sequence

Abstract

Odor sensing and learning associations of odor information to good environmental conditions is crucial for insects to find nutritious food sources and with that appropriate oviposition sites, which ensures the survival of the insects' offspring. Therefore an odor experience an insect makes during its juvenile stage may affect its behavior as an adult. It may, for example, be an advantage to prefer an oviposition site, which smells like the environment an adult fly experienced as beneficial during its own development. However, in holometabolous insects, like *Drosophila melanogaster*, whose imago stage differ in morphology and physiology, the information learned by the larva would have to survive the dramatic neuronal reconstructions of the larval nervous system during metamorphosis. If a preimaginal conditioning affects the adult behavior is therefore still controversially discussed.

In this study I examined whether the larval *Drosophila melanogaster* olfactory experience causes behavioral changes and changes in the expression of olfactory receptors (OR) in adult flies. Therefore adult *Drosophila*, which were exposed to a specific odor only during their larval stages, were tested to six different odors for their behavior compared to unconditioned control animals. I observed that indeed larval odor experience can alter adult behavior and OR expression. Surprisingly, the attraction towards an attractant (ethyl acetate) was reduced by larval experience of both the attractant itself and an unrelated odorant (limonene). This change in adult behavior was accompanied by an increase in the expression of two adult-specific ligand-binding ORs, one of them responding to ethyl acetate (Or59b), the other responding to limonene (Or19a), and the ubiquitously expressed olfactory co-receptor Orco.

The results show, that the olfactory experience larvae make can survive metamorphosis and can cause changes in the adult fly, in respect to behavior and OR expression. However, the effects I observed were rather surprising and need further investigation regarding their mechanisms and ecological implications.

Zusammenfassung

Für Insekten ist es von Vorteil, Zusammenhänge zwischen wahrgenommenen Düften und den damit auftretenden guten Umweltbedingungen zu lernen, um nährstoffreiche Futterplätze und geeignete Eiablagestellen zu finden. Dies würde das Überleben ihrer Nachkommen sichern. Aufgrund dessen könnten die Erfahrungen, die Insekten während ihrer juvenilen Phase machen, ihr Verhalten im Imaginalstadium beeinflussen. Es könnte beispielsweise von Vorteil sein, eine Eiablagestelle zu bevorzugen, welche wie die Umwelt riecht, die eine adulte Fliege während ihrer Entwicklung als positiv erfahren hat. Allerdings müssten die von den Larven gelernten Informationen in holometabolen Insekten, wie *Drosophila melanogaster*, deren Adultstadium sich in Morphologie und Physiologie von dem der Larven unterscheidet, den dramatischen neuronalen Umstrukturierungen des larvalen Nervensystems während der Metamorphose standhalten. Inwiefern eine präimaginale Konditionierung das adulte Verhalten beeinflusst, wird daher noch kontrovers diskutiert.

In dieser Studie habe ich untersucht, ob eine larvale olfaktorische Erfahrung in *Drosophila melanogaster* Verhaltensänderungen und Veränderungen der olfaktorischen Rezeptor (OR) Expression in adulten Fliegen hervorruft. Dafür wurden adulte *Drosophila*, welche einem spezifischen Duft lediglich während ihrer Larvalstadien ausgesetzt waren, auf ihr Verhalten zu sechs verschiedenen Düften im Vergleich zu unkonditionierten Kontrolltieren getestet. Dabei konnte ich beobachten, dass eine larvale Dufterfahrung tatsächlich das Verhalten und die OR Expression in Adulttieren beeinflusst. Überraschenderweise war die Anziehung zu einem Lockstoff (Ethylacetat) bei einer larvalen Erfahrung des Lockstoffes selbst und eines unabhängigen Duftstoffes (Limonen) verringert. Diese Verhaltensänderung wurde von einer Erhöhung zweier Adult-spezifischer Liganden-bindender ORs und dem ubiquitär exprimierten olfaktorischen Corezeptor Orco begleitet, wobei einer der beiden ORs auf Ethylacetat (Or59b) und der andere auf Limonen (Or19a) antwortet.

Die Ergebnisse zeigen, dass die olfaktorische Erfahrung von Larven während der Metamorphose erhalten bleibt und Veränderungen in der adulten Fliege bezüglich des Verhaltens und der OR Expression hervorruft. Jedoch sind die von mir beobachteten Effekte überraschend und bedürfen weiterer Untersuchungen zugrundeliegender Mechanismen und ökologischer Konsequenzen.

1 Introduction

The sense of chemoreception is important for organisms to cope with their environment. Therefore chemical cues from the environment, for example odors, have to be translated into neuronal information. This information is further processed and integrated in higher neuronal circuits for appropriate behavioral responses [Vosshall, 2001; Shepherd, 1994; Buck, 1996]. Most insects exhibit odor-driven behavior, with a sensitive olfactory system that is much simpler than that of vertebrates [Vosshall, 2001]. Odors occur mostly as complex mixtures in nature and differ in chemical structure, concentration and properties. Insects are able to recognize and discriminate a large number of odorants, because their olfactory system processes also the quality, quantity and intensity of different odors [Yarali et al., 2009]. This is important for finding food sources, mating partners and oviposition sites, for social interactions and avoidance of toxic and life-threatening environments [Hansson & Stensmyr, 2011; Ache & Young, 2005]

1.1 The model organism *Drosophila melanogaster* and its life cycle

Drosophila melanogaster represents a good model organism to study genetic, physiological and behavioral mechanisms of olfaction. The genome of this species is sequenced and numerous genetic tools are available that allow for specific changes in the only four chromosomes [Davis, 2005]. Many genetically defined mutants are available. Furthermore *Drosophila* breeding requires only little space and, nevertheless, the flies produce a large number of offspring, which enables the collection of a huge amount of data [Deepa et al., 2009]. *Drosophila* also have an anatomically similar olfactory system as vertebrates and there are already molecularbiological, physiological and behavioral paradigms developed for testing olfactory perception [Siddiqi, 1987; Tully, 1987; Adams et al., 2000; Vosshall, 2001; Steck et al., 2012]. Moreover fruit flies have a short temperature-dependent life cycle, which provides quick analysis of tests. At a temperature of 25°C the life cycle is completed in 10 days. After embryonic development the larvae hatch from the egg. The larvae undergo two molts, so that the complete larval phase consists of three instar stages, which take one day each. During the molts they shed the cuticle, mouth hook and spiracles. The third instar larvae pupate after three days and during that pupal stage metamorphosis takes place. The transformation processes during metamorphosis take five days, then the imago emerge

from the pupae case [Deepa et al., 2009; Carolina Biological Supply Company, Drosophila Manual]. Therefore *Drosophila melanogaster* belongs to the holometabolous insects. Thus there are differences in odor perception in the larval and adult olfactory system.

1.2 Olfactory system of adult and larval *Drosophila melanogaster*

Adult *Drosophila melanogaster* detect odor molecules at the third antennal segment as well as the maxillary palps (Fig.1 a), whereby the antennae are constantly exposed to the ambient airs with all its chemical cues [Stocker, 1994; Carlson, 1996; de Bruyne et al., 2001; Dobritsa et al., 2003; Hallem & Carlson, 2004; Hallem et al., 2004; Jefferis, 2005; Dahanukar et al., 2005; Hallem & Carlson, 2006; Hansson & Stensmyr, 2011]. The odors are detected in sensory hairs, sensilla, which are subdivided morphologically in basiconic, coeloconic and trichoid sensilla [Shanbhag et al., 1999; Shanbhag et al., 2000]. The sensilla are innervated by the dendrites of one to four olfactory receptor neurons (ORNs). Each antenna contains approximately 1200 and each maxillary palp 120 ORNs [Shanbhag et al., 1999; Shanbhag et al., 2000; Stocker, 1994; Hallem & Carlson, 2004; de Bruyne et al., 2001; Dobritsa et al., 2003; Hallem & Carlson, 2004; Jefferis, 2005]. The dendrites of the ORNs are surrounded by the fluid sensillum lymph, which also contains olfactory binding proteins (OBPs). The hydrophobic odors diffuse through pores in the cuticle of the sensilla to the sensillum lymph. There they are probably transported by OBPs through the aqueous lymph to the dendrite membrane of the ORNs [Vosshall et al., 1999; Shanbhag et al., 1999; Hansson, 2007]. Furthermore the sensillum lymph contains enzymes, which degrade the odor stimulus [Ache & Young; 2005]. The dendritic ORN membrane contains olfactory receptors (ORs) [Vosshall et al., 1999; Clyne et al., 1999; Gao et al., 1999], which are encoded by 60 different OR genes. But due to alternative splicing those 60 OR genes code for 62 OR proteins [Vosshall et al., 1999; Clyne et al., 1999; Hallem & Carlson, 2004]. The ORs belong to the G-protein coupled receptor superfamily and show a seven transmembrane domain structure, which have little sequence similarity [Vosshall et al., 1999; Clyne et al., 1999]. Depending on their chemical structure and concentration, odors activate a different number of receptors. This information is transmitted in form of action potentials (APs) with ORNs to specific brain structures. The response of the ORNs to an odor can be excitatory or inhibitory, which enables a first integration of the odor information. The ORNs show a spontaneous activity, which means a constant presence

of APs. If the AP frequency after odor stimulus rises, the ORN response is excitatory. But if after odor binding the AP frequency is lower than that of the spontaneous activity, the ORN response is inhibitory [Dahanukar et al., 2005; Hallem & Carlson, 2004].

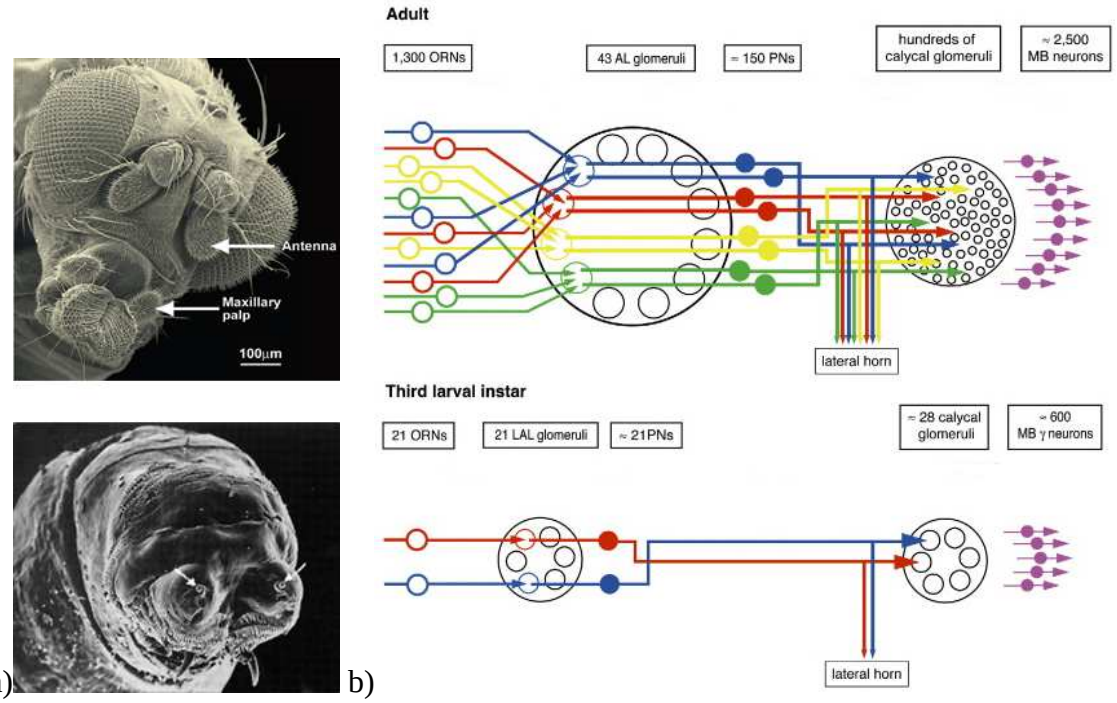


Fig.1: Olfactory system of *Drosophila melanogaster* imago and larva. a) Electron microscopy images of adult fly olfactory organs (on top)[Laissue & Vosshall, 2008] and larval olfactory organs (picture below; indicated by white arrows)[Hoare et al., 2008]. b) Adult and larval olfactory pathways. The design is similar, but the larval olfactory system shows a reduced number in the olfactory pathway architecture. ORNs, larval AL glomeruli, PNs and calycal glomeruli are related in a 1:1:1:1 fashion and therefore nearly lack cellular redundancy compared to the adult system. In the adult system the different ORN and PN types occur in multiple copies, with converging and diverging connectivity in the AL, contrary to the larval system. [modified after Ramaekers et al., 2005]

The odor information is conveyed to the antennal lobe, where ORNs expressing the same OR converge in one or few glomeruli. In those neuropil structures the ORN axons build synaptic contacts with the dendrites of projection neurons (PNs) and interneurons (INs) [Vosshall et al., 2000; Hallem & Carlson, 2004; Jefferis, 2005]. The interneurons transmit signals between the glomeruli in the antennal lobe and function either excitatory or inhibitory. The PNs provide the output from the AL to higher brain regions, the mushroom body (MB) and the lateral horn (LH) (Fig.1 b) [Hallem & Carlson, 2004; Hansson, 2007]. While the MB is mainly involved in memory formation [Heisenberg et al., 1985; de Belle & Heisenberg, 1994; Heisenberg, 1998], the lateral horn seems to code for innate odorant valence [Parnas et al., 2013]. The synaptic

contact with the PNs is formed in the calyces with Kenyon cells (KCs), which axons form a stalk to carry away the information to the output lobe [Menini, 2010; Butcher et al., 2012].

The larvae have a simpler, but adult-like olfactory system and show a strong odor evoked chemotaxis behavior [Scherer et al., 2003; Ramaekers et al., 2005]. The larval stage is therefore a good model for olfactory research and especially for olfactory learning approaches [Aceves-Pina & Quinn, 1979; Heisenberg et al., 1985; Tully et al., 1994; Dukas, 1998; Koh et al., 2000; Scherer et al., 2003]. *Drosophila* larvae have two bilaterally symmetric dorsal organs at the anterior tip of the animal (Fig.1 a), which contain 21 ORNs each. The ORNs express 25 larval *OR* genes, most of them are expressed along with the co-receptor Or83b (Orco) [Fishilevich et al., 2005]. The same as in adult flies, the first olfactory synapse is organized into glomeruli in the larval antennal lobe. Each of the 21 larval ORNs project to one of the 21 distinct larval glomeruli [Ramaekers et al., 2005]. Local interneurons establish lateral connections within the larval AL and the PNs connect the larval AL with the MB calyx and the lateral horn, which is similar to the adult stage [Marin et al., 2005]. One larval AL glomerulus is connected by PNs to one calycal glomerulus, whereby the larval MB calyces are restricted to approximately 28 glomerulus-like domains. Therefore the larval olfactory pathway is much reduced numerically compared to the adult pathway in a nonredundant way. ORNs, larval AL, glomeruli, PNs and calycal glomeruli are related in a 1:1:1:1 fashion (Fig.1 b), which makes the larvae to an elementary olfactory model system [Ramaekers et al., 2005].

1.3 ORs and IRs in larval and adult *Drosophila* and signal transduction

14 olfactory receptors of the 25 receptors expressed in larvae are larval specific, while 11 *OR* genes are expressed in both, the larval and the adult olfactory system [Fishilevish et al., 2005]. Larvae and adult flies have in common that they express most of the ORs along with Or83b (Orco), which is also evolutionary conserved in many other insects [Dahanukar et al., 2005; Vosshall & Hansson, 2011]. Orco builds heterodimers with most of the conventional ORs [Neuhaus et al., 2005; Benton et al., 2006] and is expressed in 70 – 80 % of the antennal ORNs [Larsson et al., 2004]. Contrary to the classical GPCR topology of the seven transmembrane domain receptors Orco has an inverted structure with an intracellular N-terminus and extracellular C-terminus [Lundin

et al, 2007]. Orco is associated to the ORs by evolutionary conserved intracellular loops of the co-receptor [Benton et al., 2006]. Furthermore Orco functions as a chaperone and takes part in the signal transduction [Vosshall & Hansson, 2011; Hansson & Stensmyr, 2011]. The co-receptor also connects the conventional ORs with the transport machinery of the ORNs and act therefore also as transporter, for localization and stability of the ORs in the sensory dendrites [Larsson et al., 2004; Benton et al., 2006]. Those OR/Orco heterodimers build ligand-gated non-selective cation channels, which are permeable for Na^+-, K^+- and Ca^{2+}-ions [Wicher et al, 2008; Sato et al., 2008; Smart et al., 2008; Wicher et al, 2009; Touhara et al., 2009]. This ion channel responds either ionotropic or metabotropic (Fig.2).

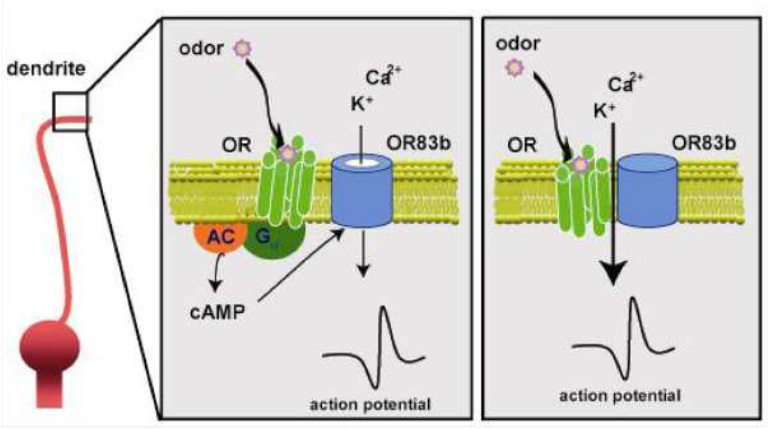


Fig.2: Olfactory activation and signal transduction of OR/Orco heterodimers. On the left side the metabotropic activation of Orco is shown, where after odor binding a G-protein activates an adenylate cyclase, which causes a higher cAMP level. cAMP activates Orco and so an influx of Na^+/Ca^{2+}-ions, which causes a depolarization of the membrane. At higher odor concentrations Orco can be directly activated by the ORs without the help of a second messenger and so ionotropic activated (image on the right side). [modified after Song et al., 2008]

The ionotropic response is fast, energy-independent and mostly in reaction to high odor-concentrations. The metabotropic response is slower, energy-dependent and more sensitive [Wicher et al., 2008; Ha & Smith, 2008; Wicher et al., 2009].

Beside the ORs, ionotropic receptors (IRs) play a crucial role in olfaction, too. They are expressed in coeloconic sensilla on the antenna. Contrary to the ORs, IRs do not form heterodimers with the co-receptor Orco. Their structure is similar to ionotropic glutamate receptors (iGluRs) and they are important for acid sensing [Benton et al., 2009; Ai et al., 2010; Silbering et al., 2011].

1.4 Preimaginal learning and memory formation

From the olfactory pathway the MB is known to play an important role in memory formation, especially long term memory, in larvae and adult *Drosophila melanogaster*. Furthermore the AL seems to mediate olfactory learning additionally to the MB [Davis, 2005]. The fruit fly has been used to study memory formation for nearly 30 years, because it provides access to genes, which are involved in the process of memory formation. Furthermore, the fundamental mechanisms in olfactory learning seem to be shared with mammals. But if and in which way olfactory memory persist during metamorphosis and influences the adult fly behavior is still not clear. Learning is defined as a change in animal behavior as a reaction to an experience. When this behavioral change persists over time it is referred to as memory, whereby the timespan of persistence of memory can be different [Davis, 2005]. There are already many olfactory learning paradigms developed, either for testing chemosensory conditioned behavior in larvae or in adult *Drosophila* [Aceves-Pina & Quinn, 1979; Heisenberg et al., 1985; Dukas, 1998; Scherer et al., 2003; Gerber & Stocker, 2007; Yarali et al., 2009]. The odor is mostly associated with an electroshock to induce aversion, or with a reward as some kind of appetitive learning. It was shown that larvae as well as adult flies are able to learn associations and change their behavior. But only few groups showed, that there seems to be a memory formation through metamorphosis, when the larvae were treated with an odor. Therefore preimaginal learning seems possible. For example Gandolfi et al. (2003) already showed that the adult response to chemical cues in parasitic wasps is influenced and increased due to preimaginal learning and that the memory persisted with a retention time of 14 days. Also Tully et al. (1994) showed that a conditioned odor avoidance in third instar larvae was still present in adult fruit flies eight days later. Thorpe (1939) already observed that adult *Drosophila*, developed from larvae reared on a peppermint-scented medium, showed a preference for perfumed medium over a non-perfumed one. On the contrary, adult flies born from larvae, which were reared in standard non-scented medium avoided the peppermint-scented medium when they got the choice to choose a non-scented one. However, other studies did not find any retention of olfactory learning through metamorphosis and therefore no preimaginal learning [Barron & Corbet, 1999].

1.5 Aim of the study

For the following study I hypothesized that an adult *Drosophila melanogaster* prefers odors from food it was reared on as a larvae. In that case, a change in the adult behavior to certain odors should be expected in respect to the kind of odor the flies experienced in their larval stages. If this is true the effect caused by an odor exposure during the larval stage has to persist during the metamorphosis of this holometabolous insect. The learned effect has to persist the anatomical transformation, which is accompanied by a drastic reorganization of the nervous system [Tissot et al., 1997]. Some neurons are only used in the larval nervous system and die during metamorphosis, others are born during larval and pupal stages to function only in adult flies. But there are also neurons functioning in the larval and adult system by reorganizing their dendrites and axons during metamorphosis [Tissot & Stocker, 2000; Marin et al., 2005]. These reorganizations are important, because the flying adult *Drosophila* have to cope with another environment than the crawling larvae.

According to my hypothesis I expected a general change in the adult behavior towards the conditioned odors, in respect to sensitivity, valence or strength in response. To examine this hypothesis the flies were reared during their larval stage in a standard food vial, which also contained a filter paper with a defined amount of a specific odor in a high concentration, which is referred to as conditioning in this work. The pupae were immediately transferred to an odor-free standard food vial, so that only the larvae were exposed to the conditioned odor. The imagos were tested for the behavioral odor response in the Flywalk. The Flywalk is a high-throughput tracking device, which enables the examination of odor evoked behavioral responses in 15 *Drosophila* individually at the same time [Steck et al., 2012].

Furthermore I hypothesized, that the exposure to a specific odor in *Drosophila melanogaster* larvae leads to expression changes of ORs in adult flies compared to untreated animals. To examine this, the RNA from antennae and maxillary palps was extracted and the relative expression of specific *OR* genes tested with the help of quantitative real-time PCR. For expression analysis flies from conditioning groups with behavioral changes were examined, as well as control group flies for comparison.

2 Methods

2.1 Chemicals

All chemicals for behavioral experiments were acquired from Sigma-Aldrich and FLUKA at the highest purity available. Fresh dilutions in mineral oil or paraffin oil were prepared once a week. (Tab.1)
Regarding materials for molecular biological studies see Appendix A.1.

2.2 Fly rearing

I used wildtype *Drosophila melanogaster* (Canton S, Bloomington), reared in standard food vials containing standard agar-cornmeal medium (recipe after E.B. Lewis, 1960). Flies were maintained under a 12h L:12h D cycle at 23-25°C and 70 % relative humidity in an incubator (Snijders Scientific, Tilburg, Netherlands).

2.2.1 Odor exposure

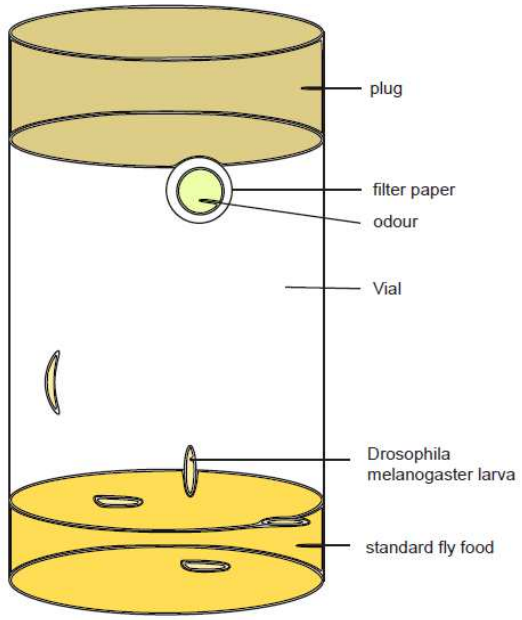
Drosophila melanogaster were exposed to a specific odor in a concentration of 10^{-1} during their whole larval stages. In order to do so 5 µl of the odor dilution was pipetted on a round piece of filter paper (Ø 1.3 cm), which was attached between the plug and the wall of the vial (Fig.3 a). To provide a continuous exposure to the odor the filter paper was exchanged daily.
I used ethyl acetate, ethyl butyrate, benzaldehyde, S-(-)-limonene, butyl acetate and hexanoic acid as conditioning odors. In parallel, a control group was reared using only 5µl of mineral/paraffin oil on a filter paper for mock conditioning.

Tab.1: List of six different odor treatment groups, which were divided into two groups for odor exposure. The exposure to ETA, ETB and BEA (odor group 1) was conducted at the same time and the display to HexA, ButA and LIM (odor group 2) took place parallel, too. As a control for both odor groups mineral oil or paraffin oil was used. (continued on the following page)

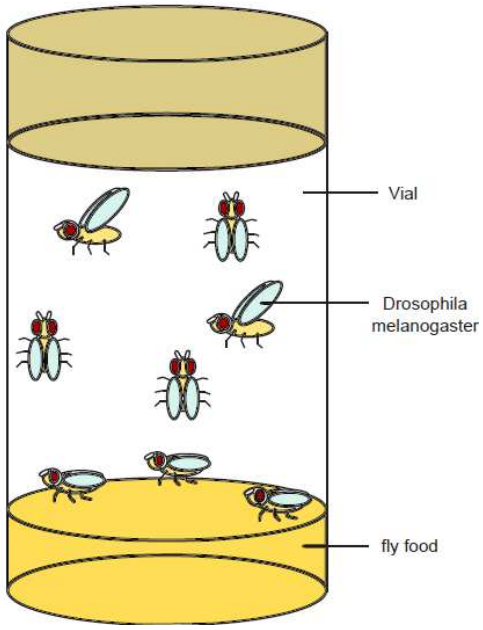
Odors	Abbreviation	CAS-Number	Company	Functional group
Ethyl acetate	ETA	141-78-6	Aldrich	Ester

Ethyl butyrate	ETB	105-54-4	SIGMA	Ester
Benzaldehyde	BEA	100-52-7	SIGMA	Aldehyde
S-(-)-Limonene	LIM	5989-54-8	Aldrich	Alkene
Butyl acetate	ButA	123-86-4	FLUKA	Ester
Hexanoic acid	HexA	142-62-1	Aldrich	Ester

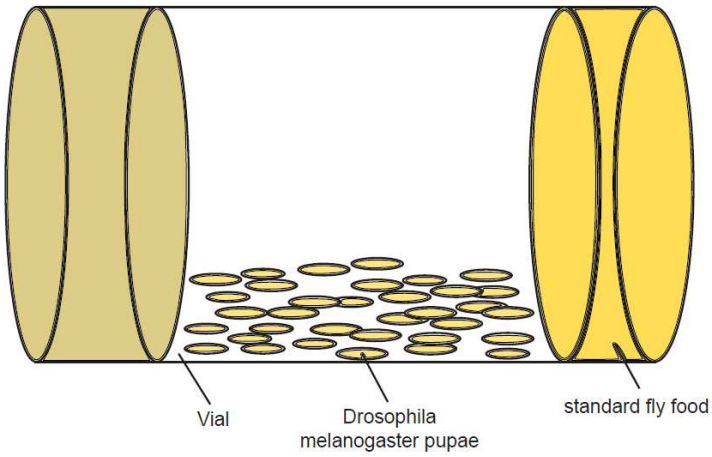
At the day of pupation, the pupae were collected carefully with a brush and transferred to another fresh food vial without any additional odor (Fig.3 b). This ensured that the animals were exposed to the odor only during their larval stages. After hatching flies were transferred to a new food vial under CO_2 anesthesia. There they were reared for 5 to 7 days for further use in the behavioral experiments (Fig.3 c). Additional conditioned female *Drosophila* which were not used in behavioral experiments were transferred into Eppendorf tubes and stored at -80°C for further molecular biological experiments.



a)



c)



b)

Fig.3: Odor treatment of *Drosophila melanogaster* larvae and rearing until adulthood. a) *Drosophila melanogaster* larvae were exposed to a specific odor in a concentration of 10^{-1} with the help of a piece of filter paper. It was loaded with 5 µl of the diluted odor, attached between the plug and the wall of the vial, and exchanged daily. b) Pupae were transferred with a brush to a new vial with standard food and no additional odor. c) Hatched *Drosophila* were transferred again to a fresh standard food vial and reared there for 5 to 7 days.

2.3 Flywalk

2.3.1 Experimental Setup

The Flywalk was used with little modifications as it was described in Steck et al. (2012). This high-throughput tracking device tests odor-evoked behavioral response of *Drosophila melanogaster*. It consists of 15 parallel glass tubes (length: 18 cm, Ø: 0.8 cm), in which individual flies are placed and allowed to distribute freely. The glass tubes are illuminated from below by an array of red light-emitting diodes (LEDs, peak emission wavelength λ = 630nm) (Fig.4 right below). As flies cannot detect light of this wave length [Yamaguchia et al., 2010], they are not distracted by the movements of neighboring flies. A 16th glass tube contains sensors for temperature and humidity within the glass tubes. The glass tubes are hermetically closed by adapters at both ends. Meshes in those adapters keep the flies from escaping the glass tubes. A humified airflow and the odor pulses are provided by an odor delivery system, which is controlled via the Software Labview 8.5 (temp.: 20 – 25°C, humidity: 70 – 75%, wind speed: 19 cm/s). For each of the existing 8 input channels to the odor delivery system (Fig.4 left side) [Olsson et al., 2011] the airflow is approximately 0.55 l/min. This results in an airflow of approximately 0.3 l/min (+/- 10 %) in each of the glass tubes. An identical airflow in all tubes is ensured by flow regulators downstream of each tube and measured by digital flowmeters connected to the downwind end of the glass tubes. The

odor pulses are well-defined in stimulus concentration, onset and duration [stimulus length: 500 ms, interstimulus interval: 90 s]. There are 8 different odor vials which can be attached to the mixing chamber of the system, so the system is able to give eight different odor pulses in a random order to avoid a learning effect. From this mixing chamber the air is piped through a split-up board to provide the 15 tubes with the same amount of air. Every odor vial contains 100 µl of a specific odor or the negative control in a 0.5 ml Eppendorf tube. Those odor vials are hermetically closed with a steel plug, a rubber-O-ring and “Input” and “Output” valves, which allow for uni-directional airflow only. The possibility of a contamination of the system is minimized by the use of the specific materials Teflon, steel and polyetheretherketone (PEEK).

The positions of the flies are tracked by an automatic tracking system, which recognizes individual flies as black dots in front of a red background (Fig.4 right at the top). The x-coordinate informs about up- and downwind movements of the flies and the y-coordinate is used to discriminate between neighboring flies. Moreover the position of each fly is stored from 15 s before until 15 s after stimulus onset. Flies which are located close to the ends of the glass tubes (i.e. outside of the “region of interest” (ROI)) are not tracked and therefore not considered in further evaluations. Besides, flies which are located at the upwind end of the tube at the time of the stimulus onset are exposed to the stimulus 1 s earlier than the flies at the downwind end of the tube. Therefore the encounter with the stimulus is individually calculated for every single fly and stimulation cycle based on the fly’s position in the tube and the speed of the stimulus.

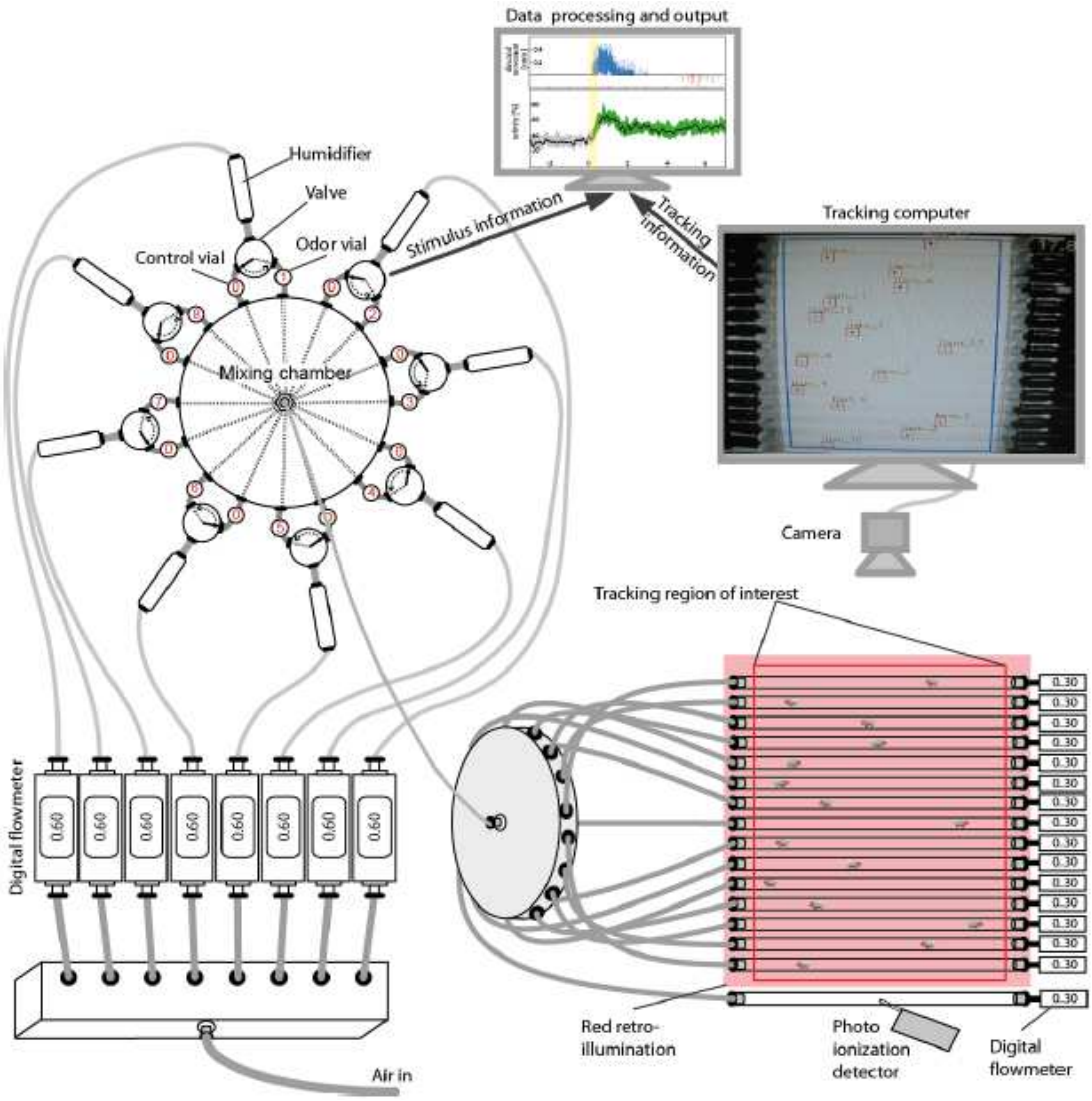


Fig.4: Schematic drawing of the Flywalk. In 15 parallel glass tubes, which are located on an array of red light-emitting diodes and Plexiglas (right below), female *Drosophila melanogaster* are individually exposed to an odor pulse every 90 s. At the downstream end of the hermetically closed glass tubes digital flowmeters are situated to control for airflow in the glass tubes. An odor delivery system (top left) [Olsson et al., 2011] provides pulses of up to 8 different odors well defined in concentration and stimulus duration. The odor delivery system is also connected to a tracking system (top right), recording the position of the fly before, during and after providing the odor pulse. To avoid any impact of the tube ends, only flies inside the "region of interest" (ROI) are recorded, with a temporal resolution of 100 ms. The odor delivery system and the tracking system are governed by a computer. [modified after Steck et al., 2012. Supplementary Information]

2.3.2 Experimental procedure

For the behavioral experiments only female *Drosophila melanogaster* were used. Irrespective of the identity of the odor used for conditioning all flies were behaviorally tested with all odors also used for conditioning in 10^{-1} and 10^{-3} dilutions. Every

experiment contained flies from different conditioning groups and flies from the control groups. Because of the amount of flies per odor treatment they had to be tested in four different groups. Flies of three out of six odor conditionings were tested with all odors either in a concentration of 10^{-1} or 10^{-3}, which make together those four groups. In every group also control animals were tested at the same time, which means two control groups per test with the same concentration.

Experiments were performed with 5 to 7 day old flies, which were food-deprived for 24 h before the start of the experiment in the late afternoon. For the experiment the flies were transferred individually to a glass tube of the Flywalk. Before the start of the experiments flies were allowed to adapt to the new environment and setup conditions for approximately 30 min.

The morning after the Flywalk run the flies which were still alive were transferred from the glass tubes in 0.5 ml Eppendorf tubes each and then put immediately in liquid nitrogen and stored at - 80°C for further use. Only the living flies were chosen, because in dead animals the mRNA is probably already degraded and therefore not suitable for molecular studies.

2.3.3 Statistical Analysis

The information of the tracking and odor delivery system is synchronized and further processed by a MATLAB Routine (The Mathworks, Naticks, USA), which also calculates the time the stimulus hits the fly in the wind channel, based on the onset and speed of the stimulus, as well as the position of the fly in the glass tube. Furthermore MATLAB interpolates the speed of movement of the flies 10 s before and 10 s after the stimulation with a temporal resolution of 100 ms.

In a given experiment, every fly was exposed to every odor approximately 40 times. Because flies sometimes left the region of interest, the number of actual tracking events per fly and odor was lower. Moreover, for some system-inherent reason, the same fly was sometimes tracked twice for a given stimulation cycle. These duplicates were removed using custom-written Macros in Microsoft Excel [Steck et al., 2012].

Further analysis was performed using custom-written routines programmed in R (www.r-project.org) [Thoma et al., 2014]. Only complete tracking events in the interval between 1 s before and 7 s after encounter with the odor pulse were considered for analysis.

The statistical analysis was conducted to make a proposition in respect to the covered distance and the time-resolved walking speed as reaction of the flies from the different treatment groups to the odor stimulations. Response time-courses of all individual flies per odor and per pulse were calculated by MATLAB. After deleting all the duplicates with Microsoft Excel, the covered distance and the walking speed of the flies were calculated in RStudio. To evaluate the walking speed at first the arithmetic mean for all time-courses of each individual fly and odor was calculated. Then for each odor treatment group the arithmetic mean of the time-courses from the arithmetic means across all individual flies in that group was analyzed. For the evaluation the reaction of 1 s before, until 7 s after the stimulus onset was taken into account.

As another metric describing the intensity of the odor response the distance a given fly covered within 4s after encountering the odor pulse was calculated for every single tracking event. From those responses the arithmetic mean covered distance of every individual fly was calculated across all tracking events per odor. To get an average group response the median of these mean covered distances across all flies in a treatment group was calculated per odor.

From those results graphs were created and statistical significances were determined with the help of the Wilcoxon signed-rank test and Anova. A p-Value < 0.05 is regarded as significant. The graphs were edited by means of the graphic design programs Adobe Illustrator CS5 and Inkscape.

The covered distance as reaction to the odor stimuli was displayed in boxplots. In the case the median response has a positive algebraic sign then the flies showed a net upwind movement. When the algebraic sign of the median response is negative then the flies showed a net downwind movement as a reaction to an odor. Furthermore the covered distance as a reaction to an odor pulse was analyzed according to the tested odor and according to the larval treatment. This double evaluation was chosen, because it enables to make different aspects of the results visible. If the data is analyzed according to the odor the differences in the response to a specific odor between the treatment groups becomes directly visible and better comparable. An evaluation according to the treatment enables the examination of a possible general change in the ranking of the odor responses (e.g. does an odor get more attractive than another one because of larval odor experience?).

The walking speed as response to the odor stimuli is shown in line plots. The goal was to look for an occurrence of a time shift in the response to the different odors between the different treatment groups.

2.4 Molecular Study

Based on the results of the behavioral experiments we decided to test, whether the expression of olfactory receptors becomes up- or down- regulated, when flies are conditioned to ethyl acetate, S-(-)-limonene, ethyl butyrate, and to the solvent control. Gene expression levels were determined for the olfactory receptors Or7a, Or19a, Or42a, Or42b, Or59b and the co-receptor Orco. For behavioral studies starvation of the flies was necessary. Starvation can potentially affect gene expression [Landis et al., 2012]. In order to exclude any effects of starvation, as a control flies were randomly chosen from the odor exposed batches, frozen pre-starvation and kept for expression analysis.

2.4.1 RNA – extraction

At first RNA from the antennae and palps of the female flies was extracted. In preparation three different methods were tested and the most suitable regarding the amount of the yielded RNA was chosen.

To isolate antennae and maxillary palps, 60 untreated wildtype female flies were transferred to a 1.5 ml Eppendorf tube and frozen in liquid nitrogen for at least 5 min. Then the tube was shaken vigorously for 15 s and immediately re-immersed in liquid nitrogen for approximately 1 min. This step was repeated 3 to 5 times to make sure that antennae and palps were separated from the body. The tubes were transferred to dry ice and 1 ml of - 20°C pre-chilled, 100 % acetone added to replace water in the tissue with acetone. The pre-chilled acetone was used to prevent RNA-degradation due to thawing. This fly/-acetone mixture was then passed through a series of dense meshes with mesh sizes chosen to retain body-parts and heads, eluting antennae and palps into a 50 ml falcon tube by using further 4 ml pre-chilled acetone. Afterwards 1.2 ml of the remaining mix was transferred to a 1.5 ml tube. Antennae and palps were spun down for 3 to 5 min at 6000 rpm and the supernatant was discarded. This step was repeated until the whole volume of filtrate was processed, with the acetone as supernatant completely removed and the tissue pelleted.

2.4.1.1 RNA - extraction with RNeasy Micro Kit (Qiagen)

350 µl Buffer RLT were added to the antennae and palps, which were then homogenized with ceramic beads (Ø 2.8 mm) in a TissueLyser LT (Qiagen) for 15 min at 50 Hz. To the homogenate 1 volume of 70 % ethanol was added and the sample transferred to an RNeasy MinElute spin column in a 2 ml collection tube. After centrifugation for 15 s at 8000 x g the flow-through was discarded, 350 µl Buffer RW1 added and centrifuged again for 15 s at 8000 x g, discarding the flow-through. After this 10 µl DNase I stock solution was mixed with 70 µl Buffer RDD and this incubation mix pipetted directly onto the RNeasy MinElute spin column membrane and incubated at RT for 15 min. After incubation 350 µl Buffer RW1 was added and centrifuged for 15 s at 8000 x g and the collection tube was discarded. The RNeasy MinElute spin column was placed in a new 2 ml collection tube and 500 µl Buffer RPE was added, then centrifuged for 15 s at 8000 x g and the flow-through discarded. In the next step 500 µl of 80 % ethanol were added to the RNeasy MinElute spin column, then the column was centrifuged for 2 min at 8000 x g and the collection tube was discarded. The RNeasy MinElute spin column was centrifuged in a new 2 ml collection tube at full speed for 5 min to dry the membrane. In the last step the RNeasy MinElute spin column was transferred in a new 1.5 ml collection tube and 14 µl RNase-free water was pipetted directly to the center of the membrane. For eluting the RNA it was centrifuged for 1 min at full speed and the filtrate was stored at -80°C.

2.4.1.2 RNA - extraction with innuPREP RNA Mini Kit (AnalytikJena)

450 µl Lysis solution RL was added to the antennae and palps, and the sample was homogenized in a TissueLyser LT (Qiagen) for 15 min at 50 Hz. The homogenate was then centrifuged for 1 min at maximum speed and the supernatant transferred to a Spin Filter D in a receiver tube. To remove genomic DNA the receiver tube was centrifuged for 2 min at 10000 x g, the Spin Filter D was discarded and an equal vol. of 70 % ethanol was added to the filtrate. For binding the RNA selectively the sample was added to a Spin Filter R in a new receiver tube and centrifuged for 2 min at 10000 x g. To wash the filter 500 µl HS was added and it was centrifuged for 1 min at 10000 x g. Then 750 µl LS was added to the Filter and centrifuged under the same conditions as before. The filtrate was discarded and the Spin Filter R with the selectively bound RNA placed in a new receiver tube, which was centrifuged for 2 min at maximum speed to remove

the ethanol. For eluting the RNA the Spin Filter R was placed in an Elution tube and 30 µl RNase-free water added to the Filter, which was incubated for 1 min at RT. After this the Elution Tube was centrifuged for 1 min at 6000 x g and the filtrate stored at -80°C.

2.4.1.3 RNA - extraction with Trizol

The antennae and palps preparation was put on ice. After adding 0.6 ml Trizol the tube was incubated for 10 min at RT and the tissue homogenized with ceramic beads (Ø 2.8 mm) for 15 min in a TissueLyser LT (Qiagen) at 50 Hz. The homogenized tissue/Trizol sample was transferred to a new tube and 72 µl of 1-Bromo-3-Chloropropane was added and mixed. The mixture was incubated for 15 to 20 min on ice and then centrifuged at 10000 x g for 15 min at 4°C. After centrifugation the upper aqueous phase was transferred to a new tube and 1/10 volume of a 10X DNAse Buffer and 1 µl of Turbo DNase was added and incubated for 30 min at 37°C for denaturing the DNA. After the DNAse treatment 0.6 ml Trizol and 72 µl of 1-Bromo-3-Chloropropane was added, mixed and incubated 15 to 20 min on ice again before the sample was centrifuged at 10000 x g for 15 min at 4°C. The complete upper aqueous phase was transferred to a new tube, 1 volume of 100 % iso-propanol added, incubated 10 min at RT and finally stored overnight at -20°C. The next day the sample was centrifuged at 12000 x g for 30 min at 4°C, the supernatant removed and the pellet washed with 0.8 ml 70 % ethanol for removing the salts. This was centrifuged for 10 min at 7500 x g at 4°C, the supernatant removed and the pellet air-dried for 5 to 10 min. After drying the pellet was re-suspended in 25 µl RNAse free water and the sample was stored at -80°C until further use.

The yield of RNA of the samples of all three used methods was determined with a photometric measurement. The RNA-extraction with Trizol resulted in 6 times more RNA compared to the other two methods. Therefore this method was further used to extract the total RNA of the antennae and maxillary palps of ETA (109 female flies), ETB (111 female flies) and LIM (106 female flies) treated flies as well as flies from both control groups (109 and 155 female flies). The extracted total RNA of those five groups was used for the cDNA-synthesis.

2.4.2 cDNA-synthesis

To synthesize cDNA from the previously extracted antennal and palp RNA the Super Script First-Strand Synthesis System for RT-PCR Kit (Invitrogen) was used, using 1 µg RNA as starting material. For each reaction 1 µg RNA, 1 µl 10 mM dNTPs, 1µl oligo(dT) primer were combined and DEPC-treated water was added to a final volume of 10 µl. This mixture was incubated for 5 min at 65°C and then placed on ice for at least 1 min. In a separate tube a mastermix with 2 µl 10X RT buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT and 1 µl RNaseOUT (40 U/µl) per reaction was prepared and 9 µl of this mix was added to each RNA/primer mixture from before. This sample was incubated for 2 min at 42°C, then 1 µl of SuperScript™ II reverse Transcriptase added to each tube and the mixture incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min and then chilled on ice. Then the reaction was collected by brief centrifugation, 1 µl of RNase H added to each tube and incubated for 20 min at 37°C. The cDNA was stored at -20°C.

2.4.3 Primer Design

To design the primer for the genes Or7a, Or19a, Or42a, Or42b, Or59b, and Orco their coding sequences (CDS) from www.flybase.org were used. Those CDS were copied to the program Geneious 6.0.5, which provides primer-3 as function. Using primer-3 primers were chosen with a target size of 20 bp long and a melting temperature (Tm) of 60°C. Primers were chosen to amplify OR fragments with a length of 150-200 bp (Tab.2).

As housekeeping genes *rp49* and for a further examination RpL13A were chosen. In case of *rp49* the primers were already present and did not have to be designed freshly. The sequences for both *rp49* primers are as follows [Strutz A., 2013. Dissertation]:

for: CCAGTCGGATCGATATGCTA
rev: TCTGTTGTCGATACCCTTGG

For the olfactory receptor 19a five different primer pairs were designed and tested, since the first primer pair designed led to unspecific amplification in the PCR. Therefore four additional primer pairs were designed and tested for correct function by sequencing the respective PCR products. Of these, primer pair 2 was chosen for further experiments.

Tab.2: For five different olfactory genes (Or7a, Or19a, Or42a, Or42b, Or59b), Orco and a house keeping gene (RpL13A) primers were designed to examine and compare their expression in the antennae and palps of the odor treated *Drosophila melanogaster* and the control groups.

Oligoname	Sequence (5´-3´)	Length [bp]	Product size [bp]	% GC	Tm [°C]
Q_Or7a_for	CCAGATGATGCTCTGCTCTG	20	200	55	59.4
Q_Or7a_rev	CTTCTCGGTGGTCATGTACG	20	200	55	59.4
Q_Or19a_for	CGAAGGTGGATTCAACGAGG	20	200	55	59.4
Q_Or19a_rev	GCAGAAAGTCTCCAGCGAAT	20	200	50	57.3
Q_Or19a_for2	TGATGTACCCCACCTGGATT	20	150	50	57.3
Q_Or19a_rev2	TGACCAGGATGAGGTAGGTG	20	150	55	59.4
Q_Or19a_for3	GCGACACTTGTCCTCAATCT	20	150	50	57.3
Q_Or19a_rev3	GTAACCAACCAGAATGGCCT	20	150	50	57.3
Q_Or19a_for4	GTGTGGAACGTAACCTTCCA	20	150	50	57.3
Q_Or19a_rev4	CATCCGACGGACATTGATCA	20	150	50	57.3
Q_Or19a_for5	CATGGTGTGGAACGTAACCT	20	150	50	57.3
Q_Or19a_rev5	CGACGGACATTGATCAGCTT	20	150	50	57.3
Q_Or42a_for	AGTTAAGCGCTTTGACGAGG	20	200	50	57.3
Q_Or42a_rev	AATTTTGGTACGGTGGCCTT	20	200	45	55.3
Q_Or42b_for	GCTAATGACGTTCGTGTGGT	20	200	50	57.3
Q_Or42b_rev	GGTCCAAAATGTTCTTGGCC	20	200	50	55.3
Q_Or59b_for	TCTGCTACACCTGCAACATG	20	200	50	57.3
Q_Or59b_rev	GAACTTGGCCACGGTTATGT	20	200	50	57.3
Orco_for	GTGCCATCAAGTACTGGGTC	20	200	55	59.4
Orco_rev	CAGCGCGTATCCTAGGTATC	20	200	55	59.4
RpL13A_for	AGCTGAACCTCTCGGGACAC	20	200	60	61.4
RpL13A_rev	CTACAAGGCAGTCCGAGGCA	20	200	60	61.4

2.4.4 Polymerase chain reaction (PCR)

Before the primers were used for the examination of the expression of the specific ORs, it was tested whether they amplified the corresponding sequences. Therefore at first PCRs were conducted with the cDNA of untreated wildtype animals and a concentration of 10 pmol/µl of the designed primer. For one PCR reaction 17.5 µl dH_2O, 2.5 µl 10X Buffer, 1.5 µl $MgCl_2$, 1 µl forward primer, 1 µl reverse primer, 0.5 µl dNTPs, 1 µl cDNA and 0.25 µl Taq polymerase were mixed together, resulting in a total volume of 25 µl. Because of the different annealing temperatures of the primers two different programs were used, at first for Or42a, Or42b and Or59b an annealing temperature of 55°C, for Or7a, Or19a and Orco an annealing temperature of 57°C. Due to unspecific amplifications the annealing temperature was changed to 60°C and the used program was the following: 94°C (3 min) as initial denaturation step, then 35 cycles with 94°C (30 sec) for denaturation, 60°C (1 min) for annealing, 72°C (1 min) for elongation, after those 35 cycles 72°C (10 min) as a final elongation step and 4°C (∞) until usage.

2.4.5 Gel Electrophoresis

To perform a gel electrophoresis with the amplification products from the PCR 1.5 % gels were prepared with 150 ml 1X TAE buffer, 2.25 g Agarose and 7.5 µl ethidium bromide. To 20 µl of the samples 4 µl 6X loading dye (New England Bioabs, Ipswich, MA) was added and as a marker a 2log Ladder (New England Biolabs) was used. The electrophoresis was performed in an electrophoresis chamber with 135 V for 30 min.

2.4.6 Gel Extraction

The gel extraction was performed with the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek). Therefore the DNA fragment of interest was excised from the gel, put in a 1.5 ml Eppendorf tube, 300 µl Binding Buffer (XP2) added, this incubated at 55°C for 10 min and vortexed every 3 min until the gel had melted. No more than 700 µl of the DNA/agarose solution was pipetted to a HiBind Mini Column in a 2 ml Collection Tube, which was centrifuged at 10000 x g for 1 min at RT and the filtrate discarded. This step was repeated until all of the DNA/agarose solution had been transferred to the column. To the collection tube 300 µl Binding Buffer (XP2) was added and this

centrifuged at maximum speed for 1 min at RT and the filtrate discarded. Then 700 µl SPW Wash Buffer was added and this centrifuged at maximum speed for 1 min at RT and the filtrate discarded. This step was repeated a second time. The empty HiBind DNA Mini Column was centrifuged for 2 min at maximum speed to dry the column matrix and after this the HiBind DNA Mini Column was transferred to a clean 1.5 ml Eppendorf tube. 30 µl Elution Buffer were added on the column membrane and incubated for 2 min at RT. To elute the DNA from the matrix it was centrifuged at maximum speed for 1 min and the DNA was then stored at -20°C until further use.

2.4.7 Cloning

2.4.7.1 Ligation

The extracted PCR fragments of the different ORs were ligated with help of the Invitrogen Dual Promoter TA Cloning Kit in the pCRII vector (Fig.5). Therefore a 5 µl ligation reaction was set up with 2.5 µl fresh PCR product, 1 µl 5X Ligation Buffer, 1 µl pCRII vector (25 ng/µl) and 0.5 µl Express Link™ T4 DNA Ligase. The ligation reaction was incubated for 15 min at RT and then stored at 4°C at least overnight.

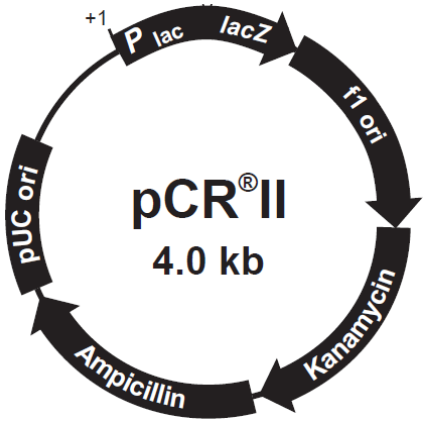


Fig.5: Structure of the used vector for ligation. Displayed is a map of the pCRII vector with insertion site of the PCR product in the lacZ gene. The vector was used for the ligation of Or7a, Or19a, Or42a, Or42b, Or59b and Orco. [modified after http://tools.lifetechnologies.com]

2.4.7.2 Transformation

For the transformation of the ligated vector DH5α™ competent *E.coli* cells were used. They were made competent following the Hanahan protocol [Sambrock & Russel, 2001]. Those chemical competent cells were thawed on ice for 5 min, then 3 µl DNA

from the ligation reaction were added and carefully mixed by stirring to avoid damaging the cells. This was incubated for 30 min on ice. After incubation a heat-shock of the cells followed for 1 min at 42°C and the cells were placed on ice for 2 min. 250 µl at RT pre-warmed SOC medium was added and the cells were incubated on a shaker for 1 h at 37°C and 225 rpm. 300 µl from each transformation were spread on a 37°C pre-warmed selective LB-agarose plate with ampicillin (100 mg per 1 l LB medium) and X-Gal (16 mg in 400 µl dimethylformamide per plate). The cells were allowed to grow colonies overnight at 37°C.

2.4.7.3 Colony PCR

After the transformation a colony-PCR was conducted to examine, which clones may contain the right PCR fragment. Therefore at least four white colonies were picked from each plate and transferred to a tube each with 50 µl LB-medium containing Ampicillin and were grown for 1 h at 37°C in a 225 rpm shaker. Then a PCR was conducted with each of the picked colonies. Therefore, with chemicals from Qiagen, 19 µl dH_2O, 2.5 µl 10X Color PCR Buffer, 0.5 µl dNTP Mix 10 mM, 1 µl M13 forward Primer (uni (-43) AGG GTT TTC CCA GTC ACG ACG TT), 1 µl M13 reverse primer (rev (-49) GAG CGG ATA ACA ATT TCA CAC AGG), 0.125 µl Taq polymerase and 2 µl LB-medium with contained *E.coli* cells were mixed for each reaction. Afterwards the PCR-product was loaded on an agarose gel and a gel electrophoresis was performed to control if they have the right length of 200 bp. Then a colony with the right fragment was chosen for the Mini preparation.

2.4.7.4 Plasmid Mini Preparation

The positive selected clones from the colony-PCR were inoculated. Therefore 5 ml LB-medium, 100 µl Ampicillin and 48 µl of the chosen colony was pipetted in a 15 ml falcon tube and incubated overnight at 37°C in a 225 rpm shaker. The overnight incubated cultures were centrifuged for 30 min at maximum speed at RT and the supernatant was discarded.

To isolate the Plasmid with the contained cDNA fragment the E.Z.N.A. Plasmid DNA Mini Kit I (Omega Bio-Tek) was used. 250 µl Solution I/RNase A was added to the cell pellet and this was mixed until complete resuspension of the cell pellet. This suspension was transferred into a new 1.5 ml Eppendorf tube, 250 µl Solution II was added and the

tube was inverted until a clear lysate occurred. 350 µl Solution III was added and the tube inverted until a flocculent white precipitate occurred. The tube was centrifuged at maximum speed for 10 min and the clear supernatant was transferred into a HiBind DNA Mini Column in a 2 ml collection tube. The collection tube was centrifuged at maximum speed for 1 min and the filtrate was discarded. 500 µl HBC Buffer were added to the Column and the tube was centrifuged at maximum speed for 1 min and the filtrate was discarded. 700 µl DNA Wash Buffer were then added to the column and the tube was centrifuged at maximum speed for 1 min and the filtrate was discarded. This step was repeated a second time. Then the empty HiBind DNA Mini Column was centrifuged for 2 min at maximum speed for drying the column matrix. The HiBind DNA Mini column was transferred to a clean 1.5 ml Eppendorf tube and 80 µl Elution Buffer was added on the column membrane. After an incubation at RT for 1 min it was centrifuged at maximum speed for 1 min to elute the plasmid DNA. The concentration of the DNA was measured with a photometer for the calculation of the needed amount of plasmid for the sequencing. The DNA was then stored at -20°C until further use.

2.4.8 Sequencing preparation and analysis

The DNA from the Plasmid Mini Preparation was either sent to MWG/Eurofin or to a house service group of the MPI for chemical ecology for sequencing.

If the DNA was sent to the house service group altogether 6 µl were needed containing 140 ng Plasmid, 0.5 µl forward or reverse primer each and sterile water for a final volume of 6 µl.

In case the samples were sent to MWG/Eurofin for sequencing, 15 µl total volume with 2 ng/µl plasmid and 2 µl forward primer were used.

The analysis of the sequences was performed with the program Geneious 6.0.5. The multiple alignments and mappings were constructed with the obtained sequences and the reference sequences from www.flybase.org to confirm that the designed primers bind to the specific ORs and so to prove that the wished product is amplified.

2.4.9 Quantitative real-time PCR (qPCR)

The qPCR was performed with the Rotor Gene SYBR Green PCR Kit (Qiagen) and the Rotor Gene Q Cycler (Qiagen). For one reaction a total volume of 10 µl is needed, with 5 µl PCR SYBR Green Master mix, 1 µl cDNA, 1 µl forward Primer, 1 µl reverse Primer and 2 µl ddH_2O. The samples were running through 95°C for 5 min, then 35 Cycles of 95°C for 5 s to denature the DNA and 60°C for 10 s as a combined annealing/elongation step. The fluorescence data collection took place at the end of the combined annealing/elongation phase. After this step the melting curve followed, which ramped up from 50°C to 99°C with 1°C every 5 s.

To examine the expression of specific ORs in ETA, ETB and LIM treated flies compared to their control groups, the previously designed primers were used. The expression levels were calculated relative to an internal control gene, *rp49*. Therefore standard curves for every used primer pair were created with the software Rotor-Gene Q Series Software 2.0.2 (Qiagen), using four dilutions up to 1:10000 from each primer pair. For preparing the standard curves cDNA from untreated flies was taken. Then the expression of the specific receptors was examined by using triplicates of a 1:10 diluted cDNA from the odor treated animals and the control flies. For every primer three qPCR runs were conducted with triplicates in each run for confirming, that technical properties do not influence the results.

The data was analyzed further with the comparative C_T method [Livak & Schmittgen, 2001; Schmittgen & Livak, 2008], whereby the calculation and relative quantification to get the threshold cycle value was done with the Rotor-Gene Q Series Software 2.0.2. This C_T-value was transferred in Microsoft Excel 2010 and the fold change of the receptor expression in the odor treated animals was calculated compared to the mineral oil treated flies. The calculation was as follows: $2^{-\Delta\Delta C_T}$= [(C_T gene of interest – C_T internal control gene)treatment group – (C_T gene of interest – C_T internal control)control group].

3 Results

This study examines, whether larval olfactory experience influences behavioral decisions of adult flies. In those cases, in which a change in the behavior could be seen, I checked, whether these changes were accompanied by changes in the expression patterns of olfactory receptors.

Drosophila larvae were exposed to one of six different odors in a concentration of 10^{-1} during all larval stages and the adult flies later tested in a behavioral assay, the Flywalk, for their reaction to all six odors and mineral oil as negative control. The responses of these conditioned flies were then compared to the responses of mock-conditioned control animals (conditioned to the solvent mineral oil). Conditioning odors were chosen to test for several hypothetical effects of larval odor experience. Ethyl acetate is a known attractant for *Drosophila melanogaster* [Monte et al., 1989; Ayyub et al., 1990; Stensmyr et al., 2003], benzaldehyde a known repellent [Rodriques & Siddiqi, 1978; Ayyub et al., 1990]. In those cases larval exposure could result in the change of odorant valence or the flies' sensitivity. The response of untreated *Drosophila* to ethyl butyrate is normally concentration-dependent, with a positive reaction to a concentration of 10^{-3} and a neutral reaction to 10^{-1}[Asahina et al., 2009; Hallem et al., 2004; Thoma et al., 2014]. In this case a previous odor exposure could lead to a sensitivity change or a habituation in the odor response. S-(-)-limonene was used, because it is a known oviposition stimulus [Dweck et al., 2013] and a conditioning may therefore cause a behavioral change in female flies. Hexanoic acid was used, because it also activates an IR compound [Ai et al., 2010], and if only in this case a behavioral change could be seen, then it could be assumed, that the change may be caused by expression changes of the IRs and not the ORs. Finally, butyl acetate was used is innately neutral to flies, independent of its concentration. Therefore, larval conditioning again could cause changes in the valence of this odor.

3.1 Flywalk

3.1.1 General behavioral response properties from control CS *Drosophila*

To analyze differences in behavioral odor reactions in conditioned flies, the odor responses of unconditioned animals have to be known for comparison. Therefore the walking speed and the covered distance as odor reaction were calculated from the data achieved after the Flywalk experiment via MATLAB. A positive algebraic sign

indicates an upwind movement of the flies after odor encounter and therefore attraction. A negative algebraic sign on the contrary shows a downwind movement of the flies and indicates a repellant response.

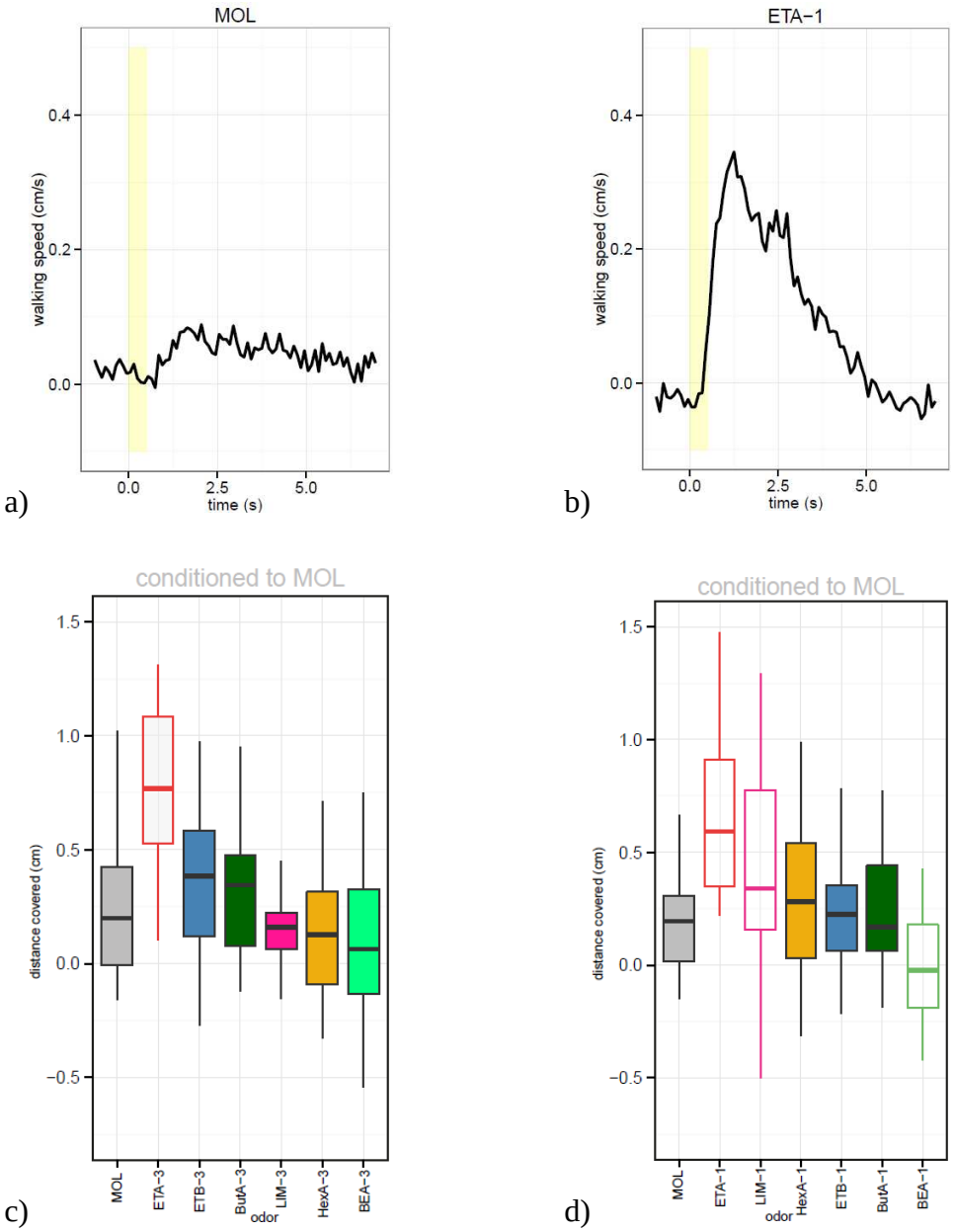


Fig.6: Response properties of mineral oil treated control animals exposed to different odors in a concentration of 10^{-3} or 10^{-1}. a) and b) Walking speed of Ctrl1 group flies as response to MOL (a) and ETA-1 (b). The yellow shaded bar shows the pulse duration of 500 ms. The reaction of the *Drosophila* was calculated 1 s before, until 7 s after odor exposure. c) Covered distance as response to six different odors at a concentration of 10^{-3} of the Ctrl1 animals. Significances compared to the MOL response are shown as unfilled boxplots ($p < 0.05$). d) Covered distance as response to six different odors at a concentration of 10^{-1} of the Ctrl1 animals is shown. Significances compared to the MOL response are shown as unfilled boxplots ($p < 0.05$). Ctrl1, N=23 (odor concentration 10^{-3}); Ctrl1, N=30 (odor concentration 10^{-1})

The mean walking speed over all flies in one group was calculated and shown from 1 s before, until 7 s after odor exposure, with a stimulus duration of 500 ms (Fig.6 a and b). With this, it was possible to get an overview of the response kinetic and strength. In Fig.6 the walking speed of the Ctrl1 group is displayed after encountering the solvent control mineral oil (a) and the odor ETA in a concentration of 10^{-1} (b). It was observed that, in contrast to a weak upwind movement in the control situation, ETA elicited a faster coordinated upwind movement in control flies. For example the Ctrl1 flies showed a maximum walking speed to ETA-1 of approximately 0.35 cm/s (Fig.6 b), but to MOL a maximum walking speed of approximately 0.08 cm/s (Fig.6 a). To get a better insight in the differences in strength of the responses the median covered distances were calculated from the mean covered distances across all flies for a time-span of 4 s after odor stimulus onset and displayed in boxplots. This enables a better comparison between the odor responses. I found only a significant attraction to ETA at both and LIM at a high concentration, while BEA was significantly repellent at high concentrations (Fig. 6c and d).

3.1.2 Changes in covered distances are odor specific

The conditioned *Drosophila melanogaster* were tested for their response to the six odors and to mineral oil as negative control in respect to their covered distance in the first 4 s after encountering an odor pulse in the Flywalk. Female flies from three different treatment groups and the control group were tested together in one run, which means three to four flies from every group.

This ensured, that all animals from different groups were really constantly tested under the same conditions. Odor conditioning was performed in two experimental blocks. In the first block flies were exposed to ethyl acetate, ethyl butyrate and benzaldehyde, the second conditioning block contained S-(-)-limonene, butyl acetate and hexanoic acid. To ensure that observed effects were caused by the odor-conditioning I mock-conditioned flies with the solvent mineral oil in both experimental blocks. Therefore all figures contain two control groups with Ctrl1 corresponding to the first block and Ctrl2 corresponding to mock-conditioned flies in the second experimental block.

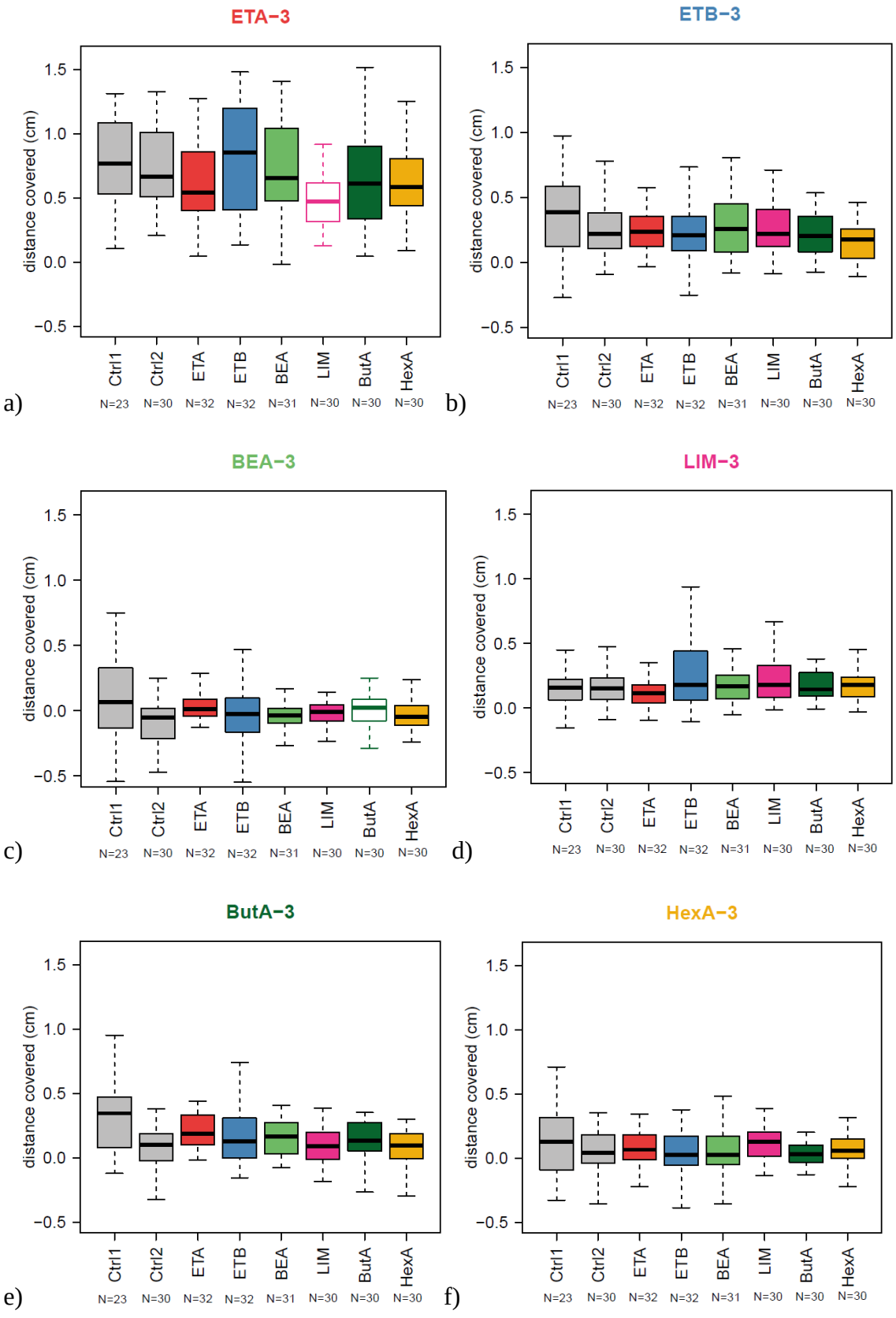


Fig.7: Boxplots with covered distances as response of the flies from six different odor conditioning groups and two control groups to the exposure with all six odors in a concentration of 10^{-3}. The unfilled plots show significances ($p < 0.05$, Wilcoxon signed-rank test) compared to the controls. The x-axis shows the different treatment groups, which are also color coded in the boxplots. ETA, ETB and BEA conditioned flies were analyzed with Ctrl1 flies and LIM, ButA and HexA flies were analyzed with the Ctrl2

group. The sample size of the different groups is displayed in every plot. Positive values show an upwind movement, negative values downwind movements. a)-f), test odorants are given on top of the graphs.

Contrary to the initial suggestions, the conditioned flies did not show a general significant change in their behavior to an odor concentration of 10^{-3} in respect to the covered distance compared to the behavior of the control groups (Fig.7).

But LIM conditioned flies responded significantly lower in respect to their covered distance after odor encounter to ETA-3 than the Ctrl2 group ($p<0.01$, Wilcoxon signed-rank test, N=30) and also compared to all the other treatment groups (Fig.7 a). This was the only group that showed a median response lower than 0.5 cm covered distance to ETA. Interestingly, also the ETA treated flies themselves showed a similar response to ETA-3 like the LIM treated flies, but the difference is not significant ($p=0.14$) compared to the control. The ETA treated flies covered a lower distance as reaction to ETA-3 than the flies of the other groups, except for the LIM group (Fig.7 a).

By exposing the *Drosophila* of the different treatment groups to the odors in a concentration of 10^{-1} a general significant change in the behavior in the conditioned flies could not be observed, either (Fig.8).

Furthermore the behavioral data was analyzed not only according to the odor, but also according to the treatment group. This evaluation provides an overview over all odor responses in one treatment group. With that I looked for changes in the ranking and therefore the preference of the odors compared to the control group. However, at both tested concentrations no changes in the ranking of the odors between the treatment groups could be observed (A.2: Fig.13; Fig.14).

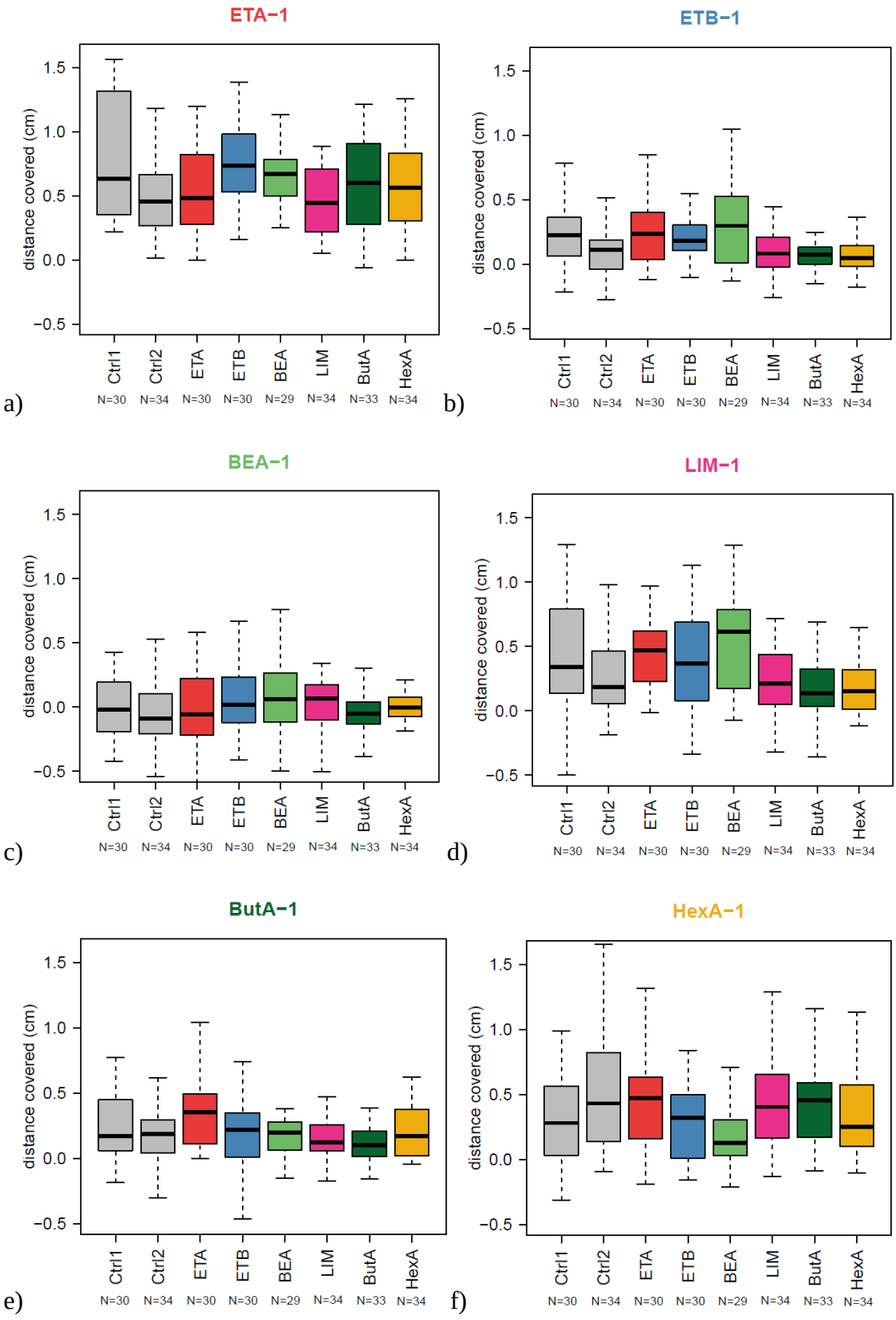


Fig.8: Boxplots with covered distance as response of the flies from six different odor conditioning groups and two control groups to the exposure with all six odors in a concentration of 10^{-1}. The unfilled plots show significances ($p < 0.05$, Wilcoxon signed-rank test) compared to the controls. The x-axis shows the different treatment groups, which are also color coded in the boxplots. ETA, ETB and BEA conditioned flies were analyzed with Ctrl1 flies and LIM, ButA and HexA flies were analyzed with the Ctrl2 group. The sample size of the different groups is displayed in every plot. Positive values

show an upwind movement, negative values downwind movements. a)-f), test odorants are given on top of the graphs.

3.1.3 The walking speed as odor response differs only in specific cases

After conducting the behavioral experiments it was also analyzed, whether flies from different conditioning groups exhibited a changed walking speed as reaction to the six tested odors and mineral oil, compared to the control animals. I tested, whether previously odor treated flies would show a shift in the response-time directly after odor encounter in relation to the control group flies. Therefore the walking speed was analyzed 1 s before, until 7 s after the odor encounter. For the same reasons as described in 3.1.2 the ETA, ETB and BEA treated flies were compared to the Ctrl1 group and the LIM, ButA and HexA treated flies were compared to the Ctrl2 group.

As it was also the case for the covered distances as odor response, there were no general changes or differences in the walking speed of the conditioned flies to the different odors in a concentration of 10^{-3} compared to their controls (Fig.9). A shift in the response-time could not be observed, either (Fig.9). However, I found differences in the walking speed as response to ETA-3. The conditioned LIM and ETA flies showed a reduced walking speed as reaction to ETA-3 compared to all other treatment groups and their specific control group (Fig.9 b and h). Furthermore the LIM treated flies showed a shorter response duration than all other groups when encountering ETA-3 (Fig.9 b and h).

When the flies of the different treatment groups were tested with the odors in a concentration of 10^{-1}, again neither a general change in the walking speed nor a change of the response latency as response could be observed, either (Fig.10). But as it could be seen at lower odor concentration there was a change in the walking speed in response to ETA-1. The ETA-conditioned flies showed a reduced maximum walking speed as response to ETA-1 compared to its control group and the other treatment groups that were tested together (Fig.10 b and h). The LIM treated flies showed a reduced maximum response to ETA-1 compared to the other treatment groups, too. But the response was similar to its control group (Fig.10 b). Furthermore the response to ETA-1 had a longer duration than to ETA-3 from flies of all treatment and control groups (Fig.9 b, Fig.10 b).

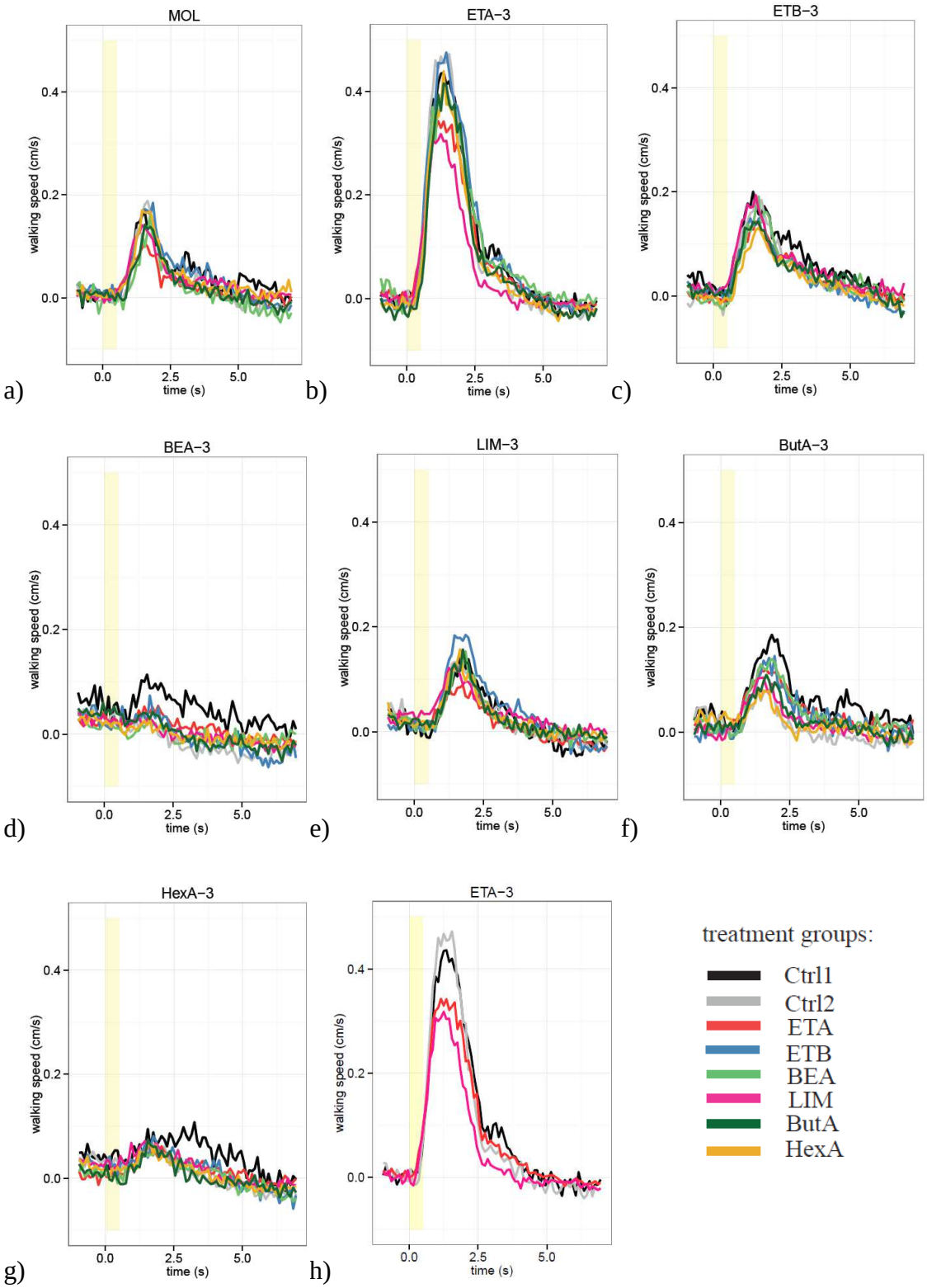


Fig.9: Walking speed of the flies from different treatment and control groups as response to an odor in the concentration 10^{-3}. Yellow shaded bar displays the odor pulse duration of 500 ms. ETA, ETB, BEA conditioned flies were tested together with Ctrl1 flies; LIM, ButA, HexA conditioned flies were tested together with Ctrl2 flies. Ctrl1, N=23; Ctrl2, N=30; ETA, N=32; ETB, N=32; BEA, N=31; LIM, N=30; ButA, N=30; HexA, N=30. a)-g), test odorants are given on top of the graphs; h) Walking speed from flies of ETA and LIM treatment and control groups as response to ETA-3.

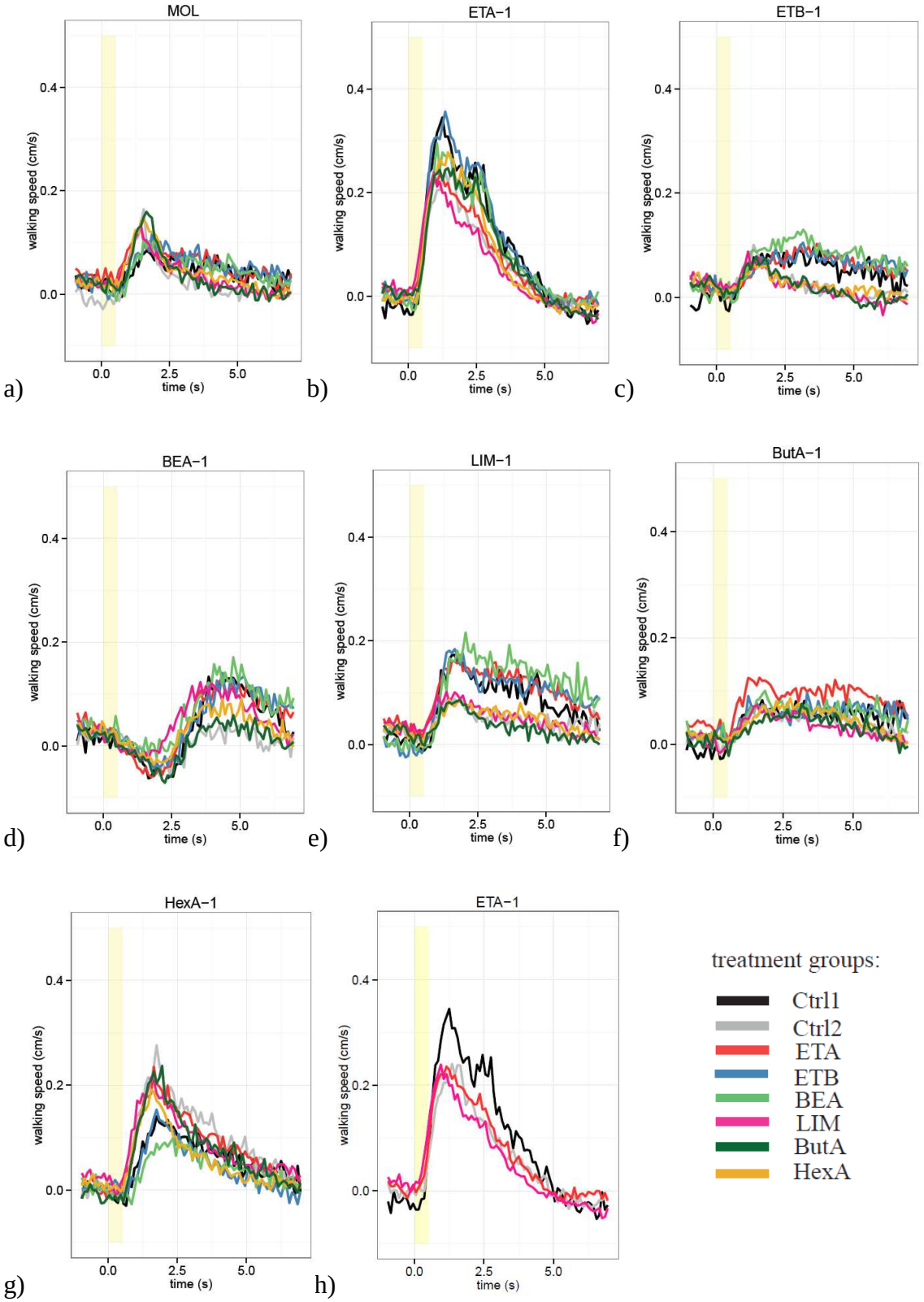


Fig.10: Walking speed of flies from different treatment and control groups as response to an odor in the concentration 10^{-1}. The yellow shaded bar displays the odor pulse duration of 500 ms. ETA, ETB, BEA conditioned flies were tested together with Ctrl1 flies; LIM, ButA, HexA conditioned flies were tested together with Ctrl2 flies. Ctrl1, N=30; Ctrl2, N=34; ETA, N=30; ETB, N=30; BEA, N=29; LIM, N=34; ButA, N=33; HexA, N=34.
a)-g), test odorants are given on top of the graphs; h) Walking speed from flies of ETA and LIM treatment and control groups as response to ETA-3.

There was also a further difference in the walking speed as response to HexA-1. Here the BEA conditioned flies showed a reduced walking speed compared to its control and all the other treatment groups (Fig.10 g). The ETA conditioned flies on the contrary had a higher walking speed to HexA-1 than the flies from its control group and the other two treatment groups, which were tested together (Fig.10 g). Moreover the ETA conditioned flies showed a higher walking speed to ButA-1 than its control group and all other treatment groups (Fig.10 f).

The results, which were obtained for the walking speed coincide with the results for the covered distance as odor response. The initial hypothesis, that general and huge behavioral changes after odor conditioning in the larval stages will occur in the adult stages towards odors, could not be supported. However I found significant effects in specific cases. The ETA and the LIM treatment group flies showed a reduced behavioral response to ETA-3 compared to all other treatment groups and their control group. The same goes for the response to ETA-1 in case of the ETA treatment group. Rather than strong general effects on overall innate odor-guided behavior, I observed pronounced odor-specific interactions which I investigated further using molecular techniques.

3.2 Molecular Biology

Specific significant changes in the behavioral response were observed in adult *Drosophila melanogaster*, when they were exposed to a specific odor during their larval stages. This change in the adult behavior might be due to a change in the expression of specific olfactory receptors that detect the respective odors in the antennae or maxillary palps of the flies. To test this hypothesis flies from the treatment groups with the strongest effects were used; these were the flies conditioned with either ETA or LIM. Furthermore the expression of specific ORs in the ETB conditioned flies was examined, since those flies exhibited a consistent response to every odor in the same way as the control group flies with no discernible difference. I expected to find OR expression changes in the flies with the strongest behavioral effects, but not in the ETB conditioned flies. As reference and for comparison flies from both control groups were also examined for OR expression.

For the examination six specific receptors were chosen, all of which respond to some of the odors we tested and are either expressed in larvae and adult *Drosophila* or only in the adult animals. The expectation was to find a change in expression of the receptors that are a) expressed in both larval and adult stage and b) for which the respective

treatment odorant was the best ligand. For the ORs expressed in larvae and adults Or7a (ligand: benzaldehyde), Or42a (ligand: ETA) and Or42b (ligands: ETA, ETB) were chosen [http://neuro.uni-konstanz.de/DoOR/default.html][Kreher et al., 2008]. The co-receptor Orco was also added, originally as control. The receptors Or19a (ligand: S-(-)-limonene) and Or59b (ligands: ETA, ETB) [http://neuro.uni-konstanz.de/DoOR/default.html] were used as receptors that are only expressed in adult flies [Kreher et al., 2008].

3.2.1 RNA-extraction and cDNA-synthesis

To examine the expression of the ORs, RNA was extracted from antennae and maxillary palps of flies from the different treatment and control groups. For this three different methods were tested and the Trizol method (see 2.4.1.3) chosen, since the yield of RNA was with Ø 179 µg/ml the highest when using the same number of flies, in comparison to the two other methods with yields of Ø 29 µg/ml (Qiagen) and Ø 18 µg/ml (AnalytikJena) RNA.
cDNA was synthesized from the extracted RNA of the different treatment and control groups, and the quality tested by PCR with primers directed against the ribosomal protein coding gene *rp49*. This gene is reported as stably expressed [Lourenco et al., 2008], and therefore suitable as a housekeeping gene. The cDNA was then used in quantitative real-time PCR to examine expression changes of the receptors.

3.2.2 Primer design, testing, cloning and sequencing

Primers for Or7a, Or19a, Or42a, Or42b, Or59b and Orco were designed. Orco was included as control since no expression changes were expected in this case. Primers were tested for sufficient specificity by qPCR products using cDNA synthesized from RNA of untreated animals, followed by TA-cloning and sequencing of the amplification products.

The sequencing results were mapped or aligned to the reference sequences of the receptors by using the map to reference tool in Geneious 6.0.5. Primers directed against *rp49* (A.3: Fig.15), Or7a (A.3: Fig.17), Or42a (A.3: Fig.23), Or42b (A.3: Fig.24), Or59b (A.3: Fig.25) and Orco (A.3: Fig.16) amplified the correct products. But in case of Or19a the sequencing result did not match the reference sequence (A.3: Fig.18). In a BLAST search the amplified sequence exhibited highest similarity to the gene

Dsim/GD22800 of *Drosophila simulans* (A.3: Fig.20), a dual oxidase. The primer pair was discarded and a new primer pair directed against Or19a designed and tested. Since Or19a and Or19b differ in only 7 bp primers against Or19a were designed, to include differences to Or19b and tested. The mapping of the obtained sequence, in case the forward primer was used, to the reference sequence of Or19a show, that it amplifies Or19a (A.3: Fig.19), but the fragment did not cover a base pair difference to Or19b. The obtained sequences from the sequencing with the reverse primer contained a base pair difference between Or19a and Or19b in the fragment, where it was shown that both receptors are amplified by using the second designed Or19a primer pair (A.3: Fig.21). It is known that Or19a is the receptor that detects LIM and several other chemicals also detected by ai2A neurons, as shown using the empty neuron system [Hallem & Carlson, 2006; Dweck et al., 2013], and therefore expression changes are likely to correspond to changes in Or19a expression. I can, however, not entirely exclude changes in Or19b expression in my dataset.

3.2.3 Expression changes of specific receptors due to odor treatment

After confirming, that the designed primers amplify the correct OR fragments, expression analyses were conducted using quantitative real-time PCR. With this method it is possible to collect data about the amplification of the amplicon during the exponential phase of the reaction run. As reporter signal the green fluorescent SYBR Green dye was used, which binds to the minor groove of the double stranded DNA. When binding to the minor groove, the intensity of the fluorescent emissions increases. Therefore, the more double stranded amplicons are produced, the more the fluorescence signal will increase. This increase in the reporter signal is directly proportional to the number of generated amplicons. For analysis of the data of the relative gene expression of Or7a, Or19a, Or42a, Or42b, Or59b and Orco the comparative C_T, $2^{-\Delta\Delta C_T}$, method was used [Schmittgen & Livak, 2008; Livak & Schmittgen, 2001]. The calculated values for the relative expression of the specific ORs and Orco represents the expression changes relative to the internal control gene *rp49* as fold change. From a 2-fold change compared to the OR expression in the control animals the expression can be considered as tendentially different. Furthermore with the $2^{-\Delta\Delta C_T}$ method the OR expression of two different treatment groups, or rather of one treatment group and its control group can be compared. In this case each of the samples has to be related to the internal control gene *rp49*.

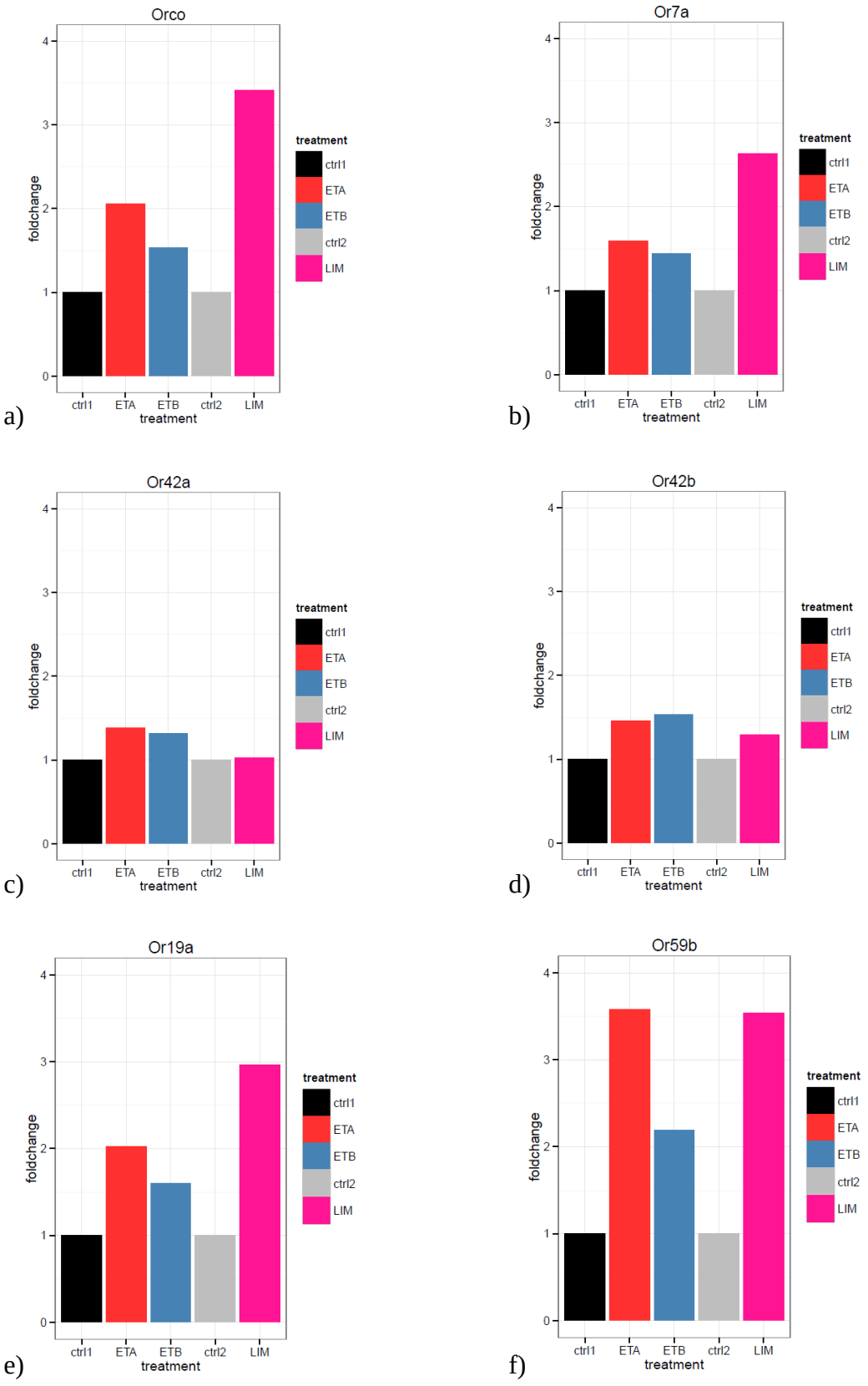


Fig.11: Relative expression of Orco, Or7a, Or19a, Or42a, Or42b and Or59b in the antennae and maxillary palps in ETA, ETB, LIM treatment group flies as fold change compared to their control groups. The data was normalized to the expression of the housekeeping gene *rp49*. The gene expression of the Ctrl1 and Ctrl2 group flies was set to 1 and the relative gene expression in the treatment groups calculated to this value with the $2^{-\Delta\Delta C}{}_{T}$ method. The gene expression of the ETA and ETB treated flies is

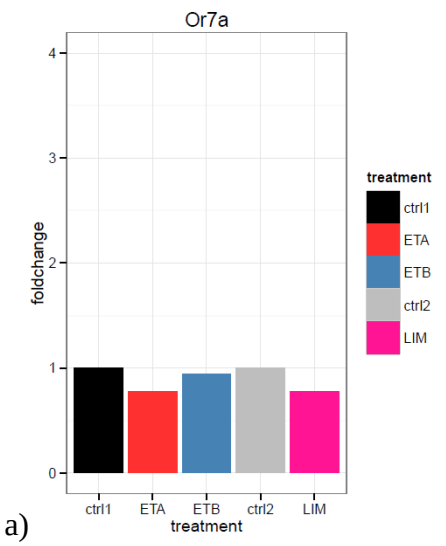
compared to the Ctrl1 group flies, the LIM treated flies were compared to the Ctrl2 group flies.
Ctrl1: N=109; Ctrl2: N=155; ETA: N=109; ETB: N=111; LIM: N=106

After optimizing PCR conditions for each primer pair, the expression of the ORs and Orco in every treatment group was analyzed with the described method, comparing with their expression in the specific control group. To minimize technical variations analysis for the ORs and Orco for the ETA, ETB, LIM , Ctrl1 and Ctrl2 group were conducted in triplicates. From those three technical replicates the average C_T value was used for further analysis (A.4: Fig.26).

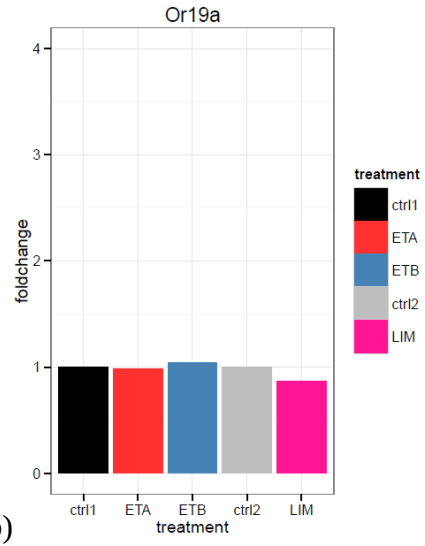
Of all examined ORs, Or19a is least expressed in the antennae and maxillary palps of the treatment and control groups, because its expression has the highest C_T value (A.4: Fig.26 d). The highest expression with the lowest C_T value is exhibited for *rp49* and Orco (A.4: Fig.26 a and b). This fits the expectation, since both will be expressed in far more neurons as each single OR.

The fold change of the specific receptors in ETA and ETB treated flies were compared to the Ctrl1 animals, the LIM treated flies on the contrary were compared to the Ctrl2 flies for the same reason as it was already described in the behavioral experiments. To facilitate comparison data was normalized so that the fold change of the Control groups was 1 (Fig.11, Fig.12). When using the housekeeping gene *rp49* as reference, expression changes were observed for three receptors out of five receptors, dependent on the treatment group (Fig.11). There is also a 3.5 fold higher expression of the co-receptor Orco in LIM conditioned flies, and a 2 fold and a 1.5 fold higher expression in ETA and ETB conditioned flies respectively when compared to their control groups (Fig.11 a). In case of Or7a the LIM conditioned flies showed a more than 2.5 fold higher expression compared to their control group, but the ETA and ETB treatment group flies only showed an expression change of around 1.5 fold, which should only be considered a tendency (Fig.11 b).

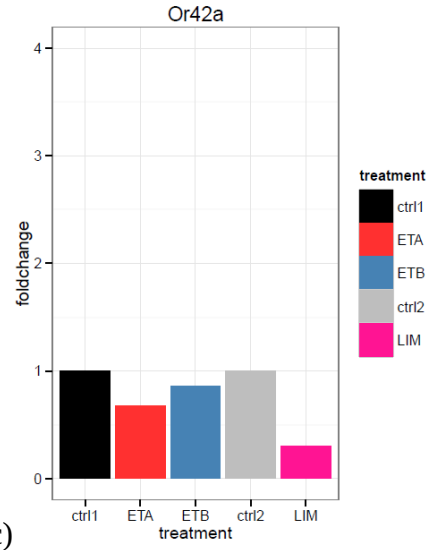
For the receptors Or42a and Or42b no considerable expression changes occurred between the treatment group flies and the control group animals (Fig.11 c and d). Or19a was nearly 3 fold higher expressed in the LIM conditioned flies than in the Ctrl2 group flies. The ETA conditioned flies have a 2 fold higher expression of Or19a and the ETB conditioned flies an around 1.6 fold higher expression then the Ctrl1 group flies (Fig.11 e). The highest expression changes were found for Or59b. There the ETA and the LIM treatment group flies had an over 3.5 fold higher expression than their both control groups.



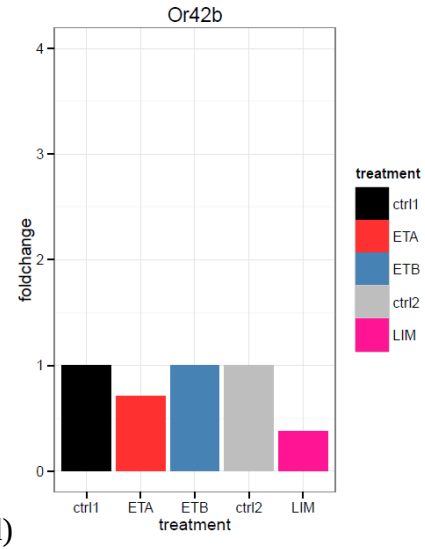
a)



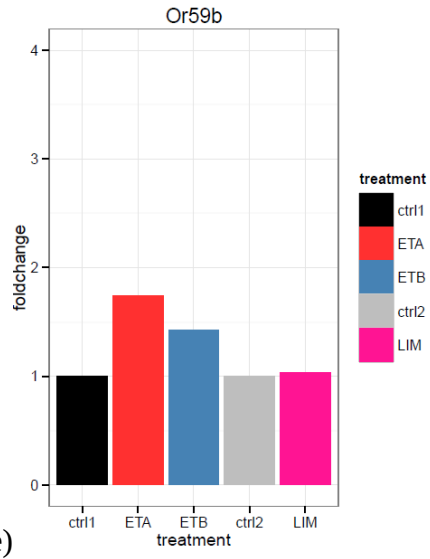
b)



c)



d)



e)

Fig.12: Relative expression of Or7a, Or19a, Or42a, Or42b and Or59b in the antennae and maxillary palps in ETA, ETB, LIM treatment group flies as fold change compared to their control groups. The data was normalized to the expression of the co-receptor Orco. Data was normalized for Ctrl1 and Ctrl2 group flies, respectively, and the relative gene expression in the treatment groups calculated to this value with the $2^{-\Delta\Delta C}{}_{T}$ method. The gene expression of the ETA and ETB treated flies is compared to the Ctrl1 group flies, the LIM treated flies have to be compared to the Ctrl2 group flies.
Ctrl1: N=109; Ctrl2: N=155; ETA: N=109; ETB: N=111; LIM: N=106

The ETB conditioned flies showed an around 2.2 fold higher Or59b expression compared to the control (Fig.11 f). That means, that the highest expression changes were observed for the receptors Or19a and Or59b, which are the two receptors only expressed in adults. Of the receptors that are expressed also in larvae only Or7a exhibited expression changes. The highest expression changes were observed in LIM conditioned flies, though the expression changes in ETA conditioned flies are also considerable.

When looking for the expression of Orco by using *rp49* as reference it was expected, that no changes occur, since Orco is not reported as differentially expressed and due to its general function unlikely to be affected. However, there are huge differences in its expression between the treatment groups and the controls (Fig.11 a).

Orco was expressed in LIM conditioned flies approximately 3.3 fold higher than in the Ctrl2 group flies. The co-receptor was furthermore 2 fold higher in ETA and 1.5 fold higher in ETB treatment group flies expressed compared to Ctrl1 group flies (Fig.11 a). If Orco and *rp49* would both not be regulated in the context of odor conditioning, there should not be any expression changes. That means that either Orco or *rp49* exhibit an expression change as response to odor conditioning in the larval stages. Therefore the data was also calculated in respect to Orco as reference, to evaluate if there expression changes occur. There were no huge expression changes in the receptors of the different treatment groups compared to the control groups observed (Fig.12), which means that Orco seems to be regulated together with the ORs. Due to the evidence in the literature that generally speaking *rp49* is stably expressed we consider it the better reference [Cardoso et al., 2014]. However to further ensure that the conditioning does not have an effect on *rp49*, additional testing using a second housekeeping gene, for example RpL13A, should be performed to validate my results.

4 Discussion

Odor sensing is a crucial ability for animals for finding nutritious food sources, mating partners, oviposition sites, for predator and toxin avoidance as well as social interactions [Hansson & Stensmyr, 2011]. It would therefore be expected, that adult animals prefer a habitat, which is similar to the environment they grew up in, when it contained exceptional living conditions. The experience an insect made as young is very likely to influence the habitat choice during its adulthood. This would also be expected for holometabolous insects like *Drosophila melanogaster*, although their imago stage differs in morphology and physiology to their larval stage and therefore also the environment they are living in varies. Hopkins already assumed in his host-selection principle in 1916 that the behavior of adult insects is conditioned by larval experience, but there is still little evidence for preimaginal conditioning and this issue is still controversially discussed [Barron, 2001]. Some groups could show that the effects of conditioning during larval stages of insects can still be observed in adulthood, others did not observe such effects. For example Gandolfi and co-workers (2003) observed that larval exposure to fruit odor in caterpillar parasitic wasps increased the adult frass response with a retention time of 14 days. Gutierrez-Ibanez and co-workers (2007) also showed that adult aphid parasitoids *Aphidius ervi* prefers vanilla odours when previously exposed to vanilla during the larval ventral opening of the mummy. Furthermore Ray (1999) showed that the fly *Musca domestica* displays a preference for the specific odor in its adulthood when exposed to this odor during the larval stages, even when using aversive odors. Tully and co-workers (1994) used *Drosophila* as model and trained third instar larvae with a combination of electroshock and a specific odor and observed that the conditioned odor avoidance was still present eight days later. On the contrary Janz and co-workers (2009) observed that the larval host plant had no effect on oviposition decisions in the adult lepidopteran *Polygonia c-album*. Also Barron & Corbet (1999) could not examine a change in the adult *Drosophila* responsiveness due to preimaginal conditioning.

4.1 Aim and accomplishment of the study and observations during tests

Therefore I was interested in, whether preimaginal odor conditioning changes responses of adult flies in respect to specific odors. If this would be the case, I furthermore investigated the biological mechanism causing the observed effect. Because there is already evidence that some individuals in insect species exhibit genetically based

variations due to learning [Dukas, 2008]. With that, I hypothesized that the olfactory experience *Drosophila melanogaster* make during their larval stages influence and change their behavior towards odors as imagos, due to changes in sensitivity or valence to the conditioned odors. For studying this *Drosophila melanogaster* larvae were conditioned to a specific odor in a defined high concentration during all three larval stages, by rearing them in a standard food vial with an additional odor-containing filter paper. This odor-containing filter paper was exchanged daily to ensure a constant exposure to the specific odor. The pupae were then immediately transferred to a fresh food vial without an additional odor to ensure an odor treatment only during the larval stages. The hatched female flies were tested at an age of 5 to 7 days for their behavioral response towards the conditioned odor, mineral oil as negative control and five further odors in the Flywalk.

Only female flies were used, because they provide a better model for behavioral analyses towards food related odors. They show a higher motivation in responding to food odors [Knaden et al., 2012], because of their need to find appropriate oviposition sites. Female flies can also be tested for their behavioral response to oviposition-related odors, like S-(-)-limonene, which I also used for conditioning. Interestingly, after the Flywalk runs I observed, that an exposure to LIM in a concentration of 10^{-1} in the test caused the female flies to lay eggs at the meshes of the adapters at the upwind site of the glass tubes, independent of the treatment group. This observation is in agreement with the finding from Dweck et al. (2013), that limonene functions as oviposition stimulus in *Drosophila* species. Moreover female flies have a larger body size than males and are therefore better accessible for further molecular biological studies, also in respect to the achieved amount of RNA.

The conducted conditioning differs from classical conditioning experiments, which are used in learning studies. There an unconditioned stimulus, for example an electroshock, is presented together with the conditioned stimulus, for example a specific odor [Alloway, 1972; Hammer & Menzel, 1995]. This way an association between conditioned and unconditioned stimulus is formed and the animal will either prefer the learned odor or avoid it, depending on the unconditioned stimulus. Such experiments are mostly performed in either larvae or adults (i.e. both conditioning and testing in the same developmental stage) and it was shown that both are able to form a memory in respect to the conditioned odor and behaved differently than non-treated animals [Quinn et al., 1974; de Belle & Heisenberg, 1994; Hammer & Menzel, 1995]. But in such classical conditioning experiments both stimuli are presented for a short timespan and

repeated several times. In contrast, I exposed the flies permanently to an odor when treated with food at the same time during all their larval stages and looked for their behavior in the adult stage. With that the flies may have formed an association between good nutritional conditions and an odor. In their natural environment the larvae are also restricted to the same place and there permanently exposed to certain habitat-specific conditions. Therefore observed changes in the experiments are likely to also occur under natural conditions. Furthermore such an experimental procedure was similarly conducted from Ray (1999) in the already mentioned preimaginal learning experiments, where a behavioral effect was observed.

4.2 Control *Drosophila* respond concentration-dependent to odors

To judge a possible behavioral change the response to the tested odors has to be known in untreated control *Drosophila melanogaster*. Untreated in this case refers to flies, which were reared in food vials together with a mineral oil containing filter paper. This ensured that the control animals were reared under the same conditions like the odor-treated animals. When testing them in the Flywalk, they showed odor-specific and concentration-dependent responses towards the six tested odors ETA, ETB, BEA, LIM, ButA and HexA. Compared to the solvent response Ctrl flies showed a faster coordinated upwind movement and a significantly higher covered distance as reaction to ETA in both tested concentrations, 10^{-1} and 10^{-3} (Fig.6 b and d). Therefore ETA can be referred to as attractant, what has also been reported in other behavioral studies [Ayyub et al., 1990; Knaden et al., 2012]. Furthermore to the high ETA concentration they responded over a longer timespan with a higher walking speed, compared to the test with a lower ETA concentration (Fig.9 b and Fig.10 b) [Thoma et al., 2014]. This observation is ecological reasonable. ETA is a common food odor [Umano et al., 1992; Hallem et al., 2004] and for finding food sources flies have to follow a concentration-gradient, the lower the distance to a food source is, the higher will be the concentration of food-related odors. Therefore a high concentration of ETA indicates a near located food source, which may enhance the motivation of flies to find the source and therefore responding longer. This may be caused by a different OR activation pattern dependent on the odor concentration. An odor can activate several ORs, for example in case of ETA activation of at least five (Or42a, Or42b, Or43b, Or47a and Or59b) and for ETB for at least eleven ORs (Or9a, Or22a, Or35a, Or42a, Or42b, Or43b, Or67a, Or67c, Or85a and Or98a) has been observed [Hallem et al., 2004; Hallem & Carlson, 2006]. With a decreasing odor concentration the number of strongly responding receptors also

decreases but by differing degrees [Hallem & Carlson, 2004]. Therefore at lower concentrations odor molecules are detected with a high selectivity, with an augmentation of the concentration the specificity decreases [Stensmyr et al., 2003]. Furthermore some highly concentrated odors may cause a strong long lasting activation of a specific receptor [Montague et al., 2011], which may cause an impairment of the signal transmission in further ORNs at glomerular level in the AL by lateral inhibition. This could be a cause for different observed concentration-dependent responses.

The responses to ETB and ButA differ also in respect to the used concentration, but not significantly. Here the Ctrl flies covered a higher distance compared to the solvent in the lower concentration (10^{-3}), but not to 10^{-1} dilution (Fig.6 c and d). The response to HexA is not significantly higher compared to the solvent, but slightly higher when testing the 10^{-1} dilution (Fig.6 c and d). In respect to BEA the Ctrl flies show a significantly reduced response to a highly concentrated odor and also a reduced response when using a 10^{-3} concentration (Fig.6 c and d). BEA can therefore be considered as repellent. Those observations are in agreement with the behavioral results of other groups doing a behavioral screening towards a set of odors [Hallem &Carlson, 2006; Asahina et al., 2009; Thoma et al., 2014].

Ctrl flies covered a significantly higher distance in upwind direction when exposed to LIM in a 10^{-1} concentration than to MOL (Fig.6 d) indicating attraction to this odor. This observation is in contrast to the results of Dweck et al. (2013), where *Drosophila* showed a neutral response to LIM. This can be explained by the different used behavioral paradigms. Compared to Trap-assays, in the Flywalk the flies are individually tested with randomized odor pulses for several hours without any distractions by other flies or light cues.

4.3 Profound behavioral changes in preimaginal conditioned *Drosophila*

4.3.1 Specific behavioral effects in respect to the covered distance

The conditioned *Drosophila melanogaster* of the six different odor treatment groups do not show a general behavioral change in the response to the six tested odors compared to their specific control groups in respect to the covered distance, neither in a concentration of 10^{-3} nor 10^{-1}, contrary to what was originally expected (Fig.7, Fig.8). That means, that due to a preimaginal odor conditioning no general change in sensitivity or valence in the odor response in the adult flies could be observed. However, some strong effects were observed in certain conditioning groups, especially in case of the

LIM and ETA conditioned flies. LIM conditioned flies covered a significantly lower distance as response to ETA in a concentration of 10^{-3} and also a lower distance towards the 10^{-1} concentrated ETA, compared to Ctrl group flies and all the other conditioning groups (Fig.7 a and Fig.8 a). ETA conditioned flies showed the same response pattern to ETA-3 and ETA-1 as the LIM conditioned flies compared to the Ctrl group flies and the other treatment groups in respect to the covered distance (Fig.7 a, Fig.8 a). Those responses were not significant, but the ETA conditioned flies covered tendentially a lower distance to ETA in both concentrations. Furthermore the ETA conditioned flies also have the tendency to respond slightly different to LIM-1 and ButA-1 compared to the controls and most of the other treatment groups (Fig.8 d and e).

The BEA conditioned *Drosophila* also showed a tendentially different response compared to the flies of the other conditioning groups in respect to LIM-1 and HexA-1 (Fig.8 d and f). This tendency was not observed in a concentration of 10^{-3} of the test odors and therefore the effects caused by an odor conditioning during larval stages seems to be also concentration-dependent.

4.3.2 Specific behavioral effects in respect to the walking speed

A general change in the walking speed as odor response of the conditioned flies was not observed, neither in a concentration of 10^{-3} nor 10^{-1} (Fig.9, Fig.10). Also a shift in the response-time after odor encounter did not occur. But as it was the case for the covered distance as odor response there are specific strong effects in the walking speed towards certain odors. The ETA- and LIM-conditioned *Drosophila* showed a reduced maximum walking speed to ETA-3 and ETA-1 compared to the controls and the other conditioning groups (Fig.9 b, Fig.10 b), which explains the shorter distance they covered after encountering the odor pulse. Moreover the LIM conditioning group showed a shorter response duration to ETA-3 (Fig.9 b and h). The ETA conditioned flies showed also a higher maximum walking speed towards HexA-1 than its Ctrl group and the two treatment groups from the same conditioning set (Fig.10 g), and a higher walking speed to ButA-1 (Fig.10 f). BEA conditioned flies on the contrary exhibited a reduced walking speed when encountering HexA-1 compared to the control and all other conditioning groups (Fig.10 g).

Interestingly, the BEA conditioned flies did not show a change in behavior towards BEA in both concentrations itself. A hypothesized change in the valence of this repellent did not occur. Thorpe (1939) showed in his preimaginal conditioning

experiment, that *Drosophila* reared on a medium containing the normally aversive odor peppermint (menthol) during their larval stage, prefer this scent over a medium without this odor as adults. Also Ray (1999) discovered that *Musca domestica* larvae reared on mint scented medium, whereby this odor functions as repellent, prefer this odor in their adulthood when tested in a two-choice test. This indicates a habituation to this repellant. That such an effect could not be seen in this study may on one hand be explained by the differences in the used behavioral assays. In the Flywalk the flies do not have the choice between two odors at the same time, they have rather to decide if they respond or not. In the assays used by Thorpe (1939) and Ray (1999) the flies have a choice between the conditioned odor and another one and with that the animals can show a preference.
On the other hand BEA is a by-product of the cyanhydric acid synthesis [North, 2003; North et al., 2008], which is also used as toxic substance against insects [Lewis, 1998. Lewis´ Dictionary of Toxicology. CRC Press LLC]. By avoiding BEA the flies avoid the toxic substance at the same time. Therefore it would be ecologically meaningful, when *Drosophila* do not learn a preference for this odor, because then they would also learn to prefer a harmful environment which reduces their fitness.

4.3.3 The behavioral responses coincide

The initial hypothesis of an occurrence of general and huge behavioral changes due to preimaginal conditioning in *Drosophila melanogaster* cannot be supported. But there is evidence of preimaginal learning effects in adult flies in specific cases, where I observed pronounced odor-specific interactions. The strongest effects were found in flies conditioned to ETA and LIM in their larval stages. Both conditioning groups showed a reduced response towards the food-related odor ETA in both tested concentrations. Interestingly those responses seem to be connected, because conditioning with those two odors independently caused the same behavioral effects, contrary to the other used odors for conditioning. That could mean, that the processing pathway of both odors is similar or strongly connected. A processing of those both odors together and a connected behavioral response would also make ecologically sense. ETA is found in many fruits that are hosts for *Drosophila* and is therefore a strong attractant [Stensmyr et al., 2003]. LIM is especially abundant in *Citrus* fruits, a preferred oviposition site of *Drosophila* [Dweck et al., 2013]. Female flies have to find an appropriate place for egg-laying on nutritious food, because their larvae are restricted to that place and therefore their survival has to be ensured beforehand. A behavioral

connection between a strong food-related odor and an oviposition-related odor could therefore be an important trait for the flies´ fitness.

The question how the larval conditioning to the one odor influences also the response to the other odor in adulthood stays to be elucidated. The memory of the odor conditioning also has to persist the massive reorganization of the larval nervous system during metamorphosis. In holometabolous insects like *Drosophila melanogaster* the larval and adult stages look very different, which is related to the different habitats they occupy and therefore the different behaviors they display. With that the metamorphosis stands for dramatic changes in the larval organization comprising the replacement of the integument and many other tissues and the histolysis of almost all muscles. Furthermore the larval nervous system and with that also the olfactory system is profoundly reorganized, whereby larval sensory neurons degenerate, with few exceptions, and are replaced by adult neurons developing from imaginal discs [Tissot & Stocker, 2000]. Most adult interneurons are formed only during metamorphosis, but many others develop from larval interneurons. This should therefore also be the case for the local interneurons which connect the glomeruli in the AL. Therefore the information of an odor from an odor conditioning in larvae may stay represented in interneuron connections and with that influence the adult behavior.

Furthermore it is known, that embryonic born PNs in the larval olfactory system are the same cells as the PNs which contribute to the much larger and more complex adult circuit. During metamorphosis each PN prunes its axon terminals and dendrites locally, leaving the main axon trunk from the cell body to the mushroom body calyx intact and extends later new processes for targeting the developing AL, MB and LH of the adult brain [Marin et al., 2005]. Therefore it would also be possible that an odor information stays present in the projection neurons, which provide a connection between the glomeruli in the AL and the MB, which is known as the main center for memory formation in insects [Heisenberg et al., 1985; Heisenberg, 1998; Armstrong et al., 1998; Davis, 2005]. MB γ neurons also prune their larva specific dendrites and axon branches before they re-extend into adult specific processes. Those MB γ neurons send their dendrites into the MB calyx and their axons into the MB axon lobes [Marin et al., 2005]. It was furthermore reported that the MB Kenyon cells survive during metamorphosis [Tissot & Stocker, 2000]. With that INs, PNs and KCs, present in the adult fly and dominant elements of the olfactory pathway and for memory formation, have in many cases an important larval component each.

Therefore it could be possible that olfactory memory is retained during metamorphosis [Tully et al., 1994; Guo & Götz, 1997; Armstrong et al., 1998; Ray, 1999] and therefore the observed specific behavioral changes in the conditioned flies are very likely a preimaginal learning effect.

4.4 Expression changes of specific ORs due to preimaginal conditioning

4.4.1 Expression analysis with only three of six conditioning groups

Metamorphosis causes also a dramatic increase in the number of receptor cells [Tissot & Stocker, 2000] and with that in the number of receptor genes, concerning the ORs from 25 in larvae to 60 in adults. Therefore I was interested in whether the observed behavioral effects are also reflected in the expression of certain ORs, known to have the conditioned and tested odors as ligands.

Furthermore I chose ORs, which are either expressed in larvae and adults, or only in adult flies. There I expected to find a possible expression change in the receptors expressed in both morphs, because only the larvae were conditioned. With that I looked for the expression of Or7a, Or42a and Or42b, which are expressed in larvae and imagos, and the expression of the adult specific receptors Or19a and Or59b [Kreher et al., 2008]. Furthermore the expression of the co-receptor Orco was analyzed in the conditioned flies in comparison to control flies, whereby no huge changes were expected and it should originally function as a control. Not all conditioning groups were used for OR expression analysis, for detecting if there even changes would occur. LIM and ETA conditioned flies were chosen for expression analysis, because they exhibited the most outstanding behavioral changes compared to their Ctrl groups. ETB conditioned flies were also examined, because they showed similar responses to every tested odor like the Ctrl flies. Therefore it would be expected that expression changes occur in LIM and ETA treated flies and the corresponding receptors, and no or little changes in the ETB conditioned flies. As reference the expression in the examined Ctrl *Drosophila* groups was analyzed.

4.4.2 Sequence similarity between Or19a and Or19b

But after sequencing of the designed receptor specific primers for expression analysis with qPCR, it was found out, that Or19a is very similar in its sequence to Or19b (A.3: Fig.21). Both receptors differ in just 7 bp, whereby 1 bp difference is positioned in an intron and should therefore not be expressed. For this reason a second primer pair was designed for Or19a including a base pair difference in the fragment. The obtained sequencing results for the second designed Or19a primer pair showed an amplification of both receptors, Or19a and Or19b (A.3: Fig.22). Therefore it cannot completely be made sure that in the conducted expression analysis only Or19a was amplified. But it is known, that Or19a respond to many odors [Hallem & Carlson, 2006], especially to the for oviposition relevant odor limonene [Dweck et al., 2013]. On the contrary, the function of Or19b remains to be elucidated. With that Or19a seems to define the response spectra of the neuron. It is known, that the Or19a and Or19b expressing ORNs target the same Glomerulus, DC1. Or19a as well as Or19b can also be found in the same ORNs [Couto et al., 2005], which respond strongly to limonene, the oviposition stimulus [Dweck et al., 2013]. With that both receptors could activate the same neuron due to conditioning and therefore lead to the observed behavioral effects. But due to the described properties of Or19a and Or19b it is more likely that Or19a is important for the observed behavioral effects and therefore the receptor, which is affected by a LIM conditioning.

4.4.3 Specific expression changes of ORs in conditioning groups

The expression of the mentioned receptors was normalized to the housekeeping gene *rp49*, and compared to the expression of the Ctrl group flies. There I observed an over 2.5 fold higher expression of Or7a in LIM conditioned flies and a 2 and 1.5 fold higher expression of this OR in ETA and ETB conditioned flies, respectively (Fig.11 b). Surprisingly, for the receptors Or42a and Or42b, which both bind ETA, no considerable expression changes were detected in the conditioned flies compared to the control flies (Fig.11 c and d). Therefore no huge expression changes were observed in the receptors, which are expressed in both larvae and adults, except for Or7a in the LIM conditioned flies. But when examining the expression of the two adult specific receptors huge expression changes were observed. Or19a was nearly 3 fold higher expressed in LIM conditioned flies, 2 fold higher expressed in ETA conditioned flies and around 1.6 fold higher expressed in the ETB conditioning group compared to their specific control

groups (Fig.11 e). The highest expression changes were found for Or59b. There, both the ETA and LIM conditioned flies showed an over 3.5 fold higher expression than their unconditioned control groups and also the ETB conditioned flies showed a 2.2 fold higher expression (Fig.11 f).

With that, the expression of both adult specific ORs seemed to be influenced the most due to preimaginal conditioning, which seems contradictory. Barron and Corbet (1999) mentioned that an odor contamination from larval conditioning during metamorphosis to the hatched adult could simulate the appearance of preimaginal conditioning. It is very unlikely that the observed behavioral and *OR* gene expression effects in this study are due to a contamination. After pupation the pupae were immediately transferred to a fresh food vial without an additional odor. The pupae were transferred with a brush, which tip was regularly cleaned with ethanol (70%) and therefore no odor residues should remain at the pupa cuticle. Directly after hatching the adult *Drosophila* were immediately transferred to a new food vial, so that they also could not come in contact with the pupa for a considerable amount of time. With that the observed effect should be due to the odor conditioning in the larval stages and in other already mentioned preimaginal conditioning studies from other groups effects were observed, too [Tully et al., 1994; Guo & Götz, 1997; Ray, 1999].
There has to be a process during metamorphosis that causes a higher expression of olfactory receptors, which are related to the odor the larvae experienced intensely. A higher expression of a receptor specific for an odor, which is related to a good experienced environment in larval stages, would possibly make adult flies more sensitive for this odor and causes them to choose a habitat with that odor over another one. But therefore there has to be a mechanism that triggers the expression regulation in newly developing ORNs during metamorphosis based on memory formation. That there is a memory retention during metamorphosis was already shown in other studies [Gandolfi et al., 2003; Ray, 1999]. Maybe the learned odor information is stored in the MB neurons and after metamorphosis the MB influences the receptor expression. But how exactly the expression of adult specific ORs can be influenced due to preimaginal conditioning remains to be elucidated.

That there are not huge expression changes in Or42a and Or42b compared to the other receptors is surprising, because they are main receptors for ETA in larvae [Kreher et al., 2008]. They are responsible for the responses to different ETA concentrations. Or42a is required for behavioral responses to a high concentration and is suggested to be a low

affinity receptor. Or42b on the contrary is required for behavioral responses to a low concentration and is suggested to be a high affinity receptor [Kreher et al., 2008]. With those two receptors larvae already can respond to a broad range of ETA concentrations [Kreher et al., 2008]. With that an ETA conditioning should very likely also influence the expression of those two receptors. But in an experiment from Kreher et al. (2008), where larvae only had one functional neuron expressing Or42a, they were still able to respond to a subset of stimuli. That means that this receptor also recognize a broader range of odors, but also that the larval behavior depends on a combined input from multiple ORs. This may be the cause why a conditioning to only ETA does not induce large expression changes of this OR.

The highest expression changes were observed for LIM conditioned flies and also considerable are the expression changes in ETA conditioned flies, which coincides with the observations made in the behavioral tests. There also the strongest different effects compared to the Ctrl group flies were seen in these two conditioning groups. Also that the strongest expression changes were found for the LIM specific Or19a and the ETA binding Or59b for both conditionings indicates a connection between the ETA and LIM processing as it was already mentioned in the behavioral results.

4.4.4 Orco expression changes due to preimaginal conditioning

When looking for the expression of Orco in the conditioning groups compared to the control groups, surprisingly strong changes were observed. Orco was approximately 3.3 fold higher expressed in LIM conditioned flies, 2 fold higher expressed in ETA conditioned flies and 1.5 fold higher expressed in ETB treatment group flies (Fig.11 a). This indicates that the expression of the co-receptor Orco is regulated together with the expression of the ORs as a result of preimaginal conditioning and does not stay stable. This would make sense, because most of the ORs heterodimerize with Orco and when more receptor molecules are needed, automatically more Orco molecules would be needed, too for building a functional olfactory receptor. Furthermore Orco functions as chaperone and is therefore necessary to localize the ORs to the dendritic membranes of the ORNs and with that essential for odorant detection [Larsson et al., 2004; Benton et al. 2006]. Thus, it would be plausible if the Orco expression changes, too with a changed OR expression. But this has to be further elucidated.

The Orco expression was also normalized to the housekeeping gene *rp49*. The observed huge changes in the Orco expression, which was originally thought as control, could also indicate a regulation of *rp49* due to the conditioning and not of Orco. But this is very unlikely, because *rp49* was already shown to be stably expressed by others [Cardoso et al., 2014]. Furthermore *rp49* is a ribosomal protein and a conditioning with an odor is more likely to influence an olfaction-related co-receptor, than a protein taking part in many different biochemical pathways. But to validate the obtained expression results also in respect to the other receptors, the expression was also normalized to Orco and not to *rp49*. If Orco is regulated together with the ORs due to conditioning, then no huge expression changes should occur compared to a normalization with *rp49*. Indeed, there were no huge expression changes observed in the receptors of the different conditioning groups compared to the control groups when it was normalized to Orco (Fig.12). With that the explained changes in the receptor expression in the conditionings are valid. The little differences in expression when normalized to Orco in the LIM conditioned flies may also indicate that the Orco expression is regulated together with the OR expression, but not 1:1, because Orco also has other functions and there are also ORs that do not dimerize with the co-receptor. But to support this, another housekeeping gene should be used for normalization and the achieved data compared to the results from the *rp49* and the Orco normalization. There the observed expression changes should be the same, as when using *rp49* as reference. The primers for the housekeeping gene RpL13A were already designed but did not amplify the desired product. Therefore this remains to be done.

For some odorants it is known that they are toxic at high concentrations. Therefore it could be implied that an odor conditioning with a concentration of 10^{-1} may poison the larvae and cause different behavioral effects. That is very unlikely for the conducted study because the observed effects are very specific and during the odor exposure in the larvae stages the larvae also crawled directly to the filter paper and even started to pupate there, and were not repelled by the odor (A.5: Fig.27).

4.5 Summary

In summary, in the behavioral experiments it was observed that adult *Drosophila melanogaster* change their behavior towards odors, when they are exposed to a specific odor during their larval stages. An expected general change in the adult behavior towards the conditioned odors, in respect to sensitivity, valence or strength in response

could not be observed, but instead strong odor-specific interactions, whereby the strongest effects were provided by the LIM and ETA conditioned flies. The behavioral results were rather unexpected, for example the ETA conditioned flies showed a reduced attractant response towards ETA itself compared to the control flies and the other conditioning groups, except for the LIM conditioning group. This means, that a food-related odor experienced intensely by the larvae becomes less attractive in adults. This may mean that the larval odor experience is not that important for adult flies for foraging. It may, however, be important for oviposition, because a female fly´s successful development to the adult stage may be indicative of a favorable environment for its own offspring.

Furthermore the obtained results support my second hypothesis, that the exposure to a specific odor in *Drosophila melanogaster* larvae leads to expression changes of ORs in adult flies compared to untreated animals. Here the effects were also receptor specific and the highest changes were observed in LIM and ETA conditioned flies, which coincides with the behavioral results.

4.6 Outlook

It has to be mentioned that the expression analysis was conducted with the same set of flies, but from different conditioning trials. To validate the observed expression changes of specific ORs in conditioned flies compared to the control groups further biological replicates are needed, as well as the test with the already mentioned second housekeeping gene to support *rp49* as reference. Furthermore with the obtained data it remains to be elucidated if the higher expression of some ORs is due to an induced change in the number of ORNs or the result of a higher number of receptors in one specific ORN. A change in the ORN number could be tested by expressing the green fluorescent protein GFP together with a specific receptor where I examined expression changes, i.e. Or59b, via the Gal4/UAS-system [Brand & Perrimon, 1993; Rosenzweig, 2005] and condition the flies with the best ligand (odor) for the used receptor. After conditioning, the number of green fluorescent ORNs has to be counted and compared to the number of the specific receptor-expressing ORNs from untreated flies. In case a change in the number of ORNs cannot be observed it is very likely that the observed higher expression of some ORs in conditioned flies is due to a change in the receptor number per ORN.

Furthermore it could be examined, whether a permanent activation of the specific receptor neurons cause the same observed behavioral effects, for example by expressing the temperature-sensitive cation channel dTRPA1 in specific ORN populations via the Gal4/UAS-system [Brand & Perrimon, 1993; Rosenzweig, 2005]. By rearing the flies over a temperature of 26°C the ORN should be constantly activated. This simulates an odor conditioning with the ligand for the specific receptor expression in this ORN, because during conditioning specific receptors are constantly activated by the used odor, too. Such an approach would on the one hand elucidate, whether larval neuronal activity is sufficient to lead to the observed expression changes. On the other hand it would also be helpful to exclude the contamination of pupal cases as a possible source of the behavioral changes in the adult fly.

Furthermore the expression analysis could also be conducted in the other three conditioning groups, because there also small specific effects occurred, and so it could also be examined if the observed expression changes are a general phenomenon in respect to the combination of used odor and receptor.

The conducted behavioral Flywalk experiment was a no-choice assay, therefore it is not possible to make a statement about the odor preferences of the conditioned flies, or rather, if they would prefer food in addition to the conditioned odor over food without that odor. For a preference test, a choice assay would have to be conducted, what should also be possible by using the Flywalk. There they can be exposed via a defined stimulus with the headspace of standard food and the headspace of standard food with contained additional odor of the conditioning. Both stimuli differ only in the presence of the conditioned odor. A stronger upwind response towards one of both stimuli could therefore be considered as preference.

Moreover it would be interesting to look for the behavior and expression changes of the F1 generation of the conditioning groups compared to the control groups, if the preimaginal learned effects are inherited due to epigenetic changes.

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Appendix

A.1 Material for expression analysis

A.1.2 Chemicals and consumables

Name	Distributor
1-Bromo-3-chloropropane	Sigma-Aldrich, St. Louis/ USA
10X DNAse buffer	Ambion INC, Austin/TX
Acetic acid	ROTH GmbH & Co, Karlsruhe/ G
Acetone	ROTH GmbH & Co, Karlsruhe/ G
Agar agar	ROTH GmbH & Co, Karlsruhe/ G
LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf/ G
Ampicillin	ROTH GmbH & Co, Karlsruhe/ G
Bacto- tryptone	ROTH GmbH & Co, Karlsruhe/ G
Bacto- yeast extract	ROTH GmbH & Co, Karlsruhe/ G
Bromophenole blue	ROTH GmbH & Co, Karlsruhe/ G
Diethylpyrocarbonate/DEPC	Sigma-Aldrich, St. Louis/ USA
dNTPs, Roti®-Mix PCR 3	ROTH GmbH & Co, Karlsruhe / G
Ethanol	ROTH GmbH & Co, Karlsruhe/ G
Ethidium bromide	ROTH GmbH & Co, Karlsruhe/ G
Iso-propanol	ROTH GmbH & Co, Karlsruhe/ G
$MgCl_2$	QIAGEN, Hilden/ G
TRI Reagent	Sigma-Aldrich, St. Louis/ USA
TRIS	ROTH GmbH & Co, Karlsruhe/ G
X-Gal	ROTH GmbH & Co, Karlsruhe/ G

A.1.3 Antibiotics

Ampicillin (50mg/ml)	ROTH GmbH & Co, Karlsruhe/ G

A.1.4 Enzymes

Express Link™ T4 DNA Ligase	Invitrogen, Darmstadt/ G

HotStar Taq Plus DNA Polymerase	QIAGEN, Hilden/ G
SuperScript II reverse Transcriptase	Invitrogen, Darmstadt/ G
Taq DNA Polymerase	QIAGEN, Hilden/ G
Turbo DNAse	Ambion INC, Austin/TX

A.1.5 DNA ladder/ size marker

2-Log DNA Ladder (0.1–10.0 kb)	New Englang BioLabs, Frankfurt a. M. / G

A.1.6 Kits

Name	Distributor
E.Z.N.A Gel Extraction Kit	Omega Bio- Tek, VWR International GmbH, Darmstadt/ G
E.Z.N.A Plasmid DNA Mini Kit I	Omega Bio- Tek, VWR International GmbH, Darmstadt/ G
innuPREP RNA Mini Kit	AnalytikJena, Jena/ G
RNeasy Micro Kit	QIAGEN, Hilden/ G
Rotor Gene SYBR Green PCR Kit	QIAGEN, Hilden/ G
SuperScript First-Strand Synthesis System for RT-PCR	Invitrogen, Darmstadt/ G
Dual Promoter TA Cloning Kit	Invitrogen, Darmstadt/ G
Taq DNA Polymerase Kit (5000)	QIAGEN, Hilden/ G

A.1.7 Cloning vector

pCRII vector	Invitrogen, Darmstadt/ G

A.1.8 Bacterial strain

DH5α™ Competent E.coli cells	Culture of E.coli was donated by HKI, Jena/ G

A.1.9 Buffers and solutions

6× loading dye (10ml): 3ml glycerol (100%), 7ml dH_2O, 2.5 mg Bromophenole blue

50x TAE-buffer (1l):	242g TRIS base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA (pH 8.0), fill up to 1l with dH_2O
1x TAE-buffer (5l):	100 ml 50x TAE-buffer, fill up to 5l with dH_2O
Agarose gel (1.5 %):	150 ml TAE buffer, 2.25 g Agarose, 7.5 µl ethidium bromide

A.1.10 Culture media

LB-medium (lysogeny broth medium) 1l:	10g Bacto-tryptone, 10g NaCl, 5g Bacto-yeast extract, Fill up to 1l with dH_2O, pH 7.5 (NaOH) Stored at RT
LB_{Amp}–medium (100ml):	LB-medium + 100 µl ampicillin Stored at 4°C
LB_{Amp}-Agar (100ml):	LB - medium + 1.5 g Agar agar To dissolve the Agar agar the mixture had to be cooked in a microwave. + 60µl ampicillin [50mg/ml] poured in petri plates (10 cm diameter) Stored at 4°C
S.O.C.-medium:	compounded after recipe from D.Hanahan, 1983

A.1.11 Laboratory equipment

Besides the general laboratory equipment, following utensils were used:

Centrifuge Type 5810 R	Eppendorf, Hamburg/ G
Centrifuge Type 5415 R	Eppendorf, Hamburg/ G
Comfort Thermomixer 1.5 ml and 2 ml	Eppendorf, Hamburg/ G
Electrophoresis system, Mupid-exU	Advance, Potsdam/ G
Gel documentation, Bio - Vision	Peqlab, Erlangen/ G
Incubator, Kendro B12 FunctionLine	Heraeus Instruments, Hanau/ G
Rotary incubator	HT, Bottmigen/ CH
Rotor Gene Q Cycler	QIAGEN, Hilden/ G
Spectrometer, BioPhotometer	Eppendorf, Hamburg/ G
Thermal cycler, GeneAmp PCR System 9700	Applied Biosystems, Darmstadt/ G
TissueLyser LT	QIAGEN, Hilden/ G

A.1.12 Software

Adobe Illustrator CS5	Adobe Systems GmbH, München/ G
Geneious 6.0.5	Biomatters, Auckland/NZ
Inkscape	http://www.inkscape.org/de/
MATLAB	The Mathworks, Naticks/ USA
Rotor Gene Q Series Software 2.0.2	QIAGEN, Hilden/ G
RSudio	http://www.r-project.org/

A.2 Covered distance according to treatment groups

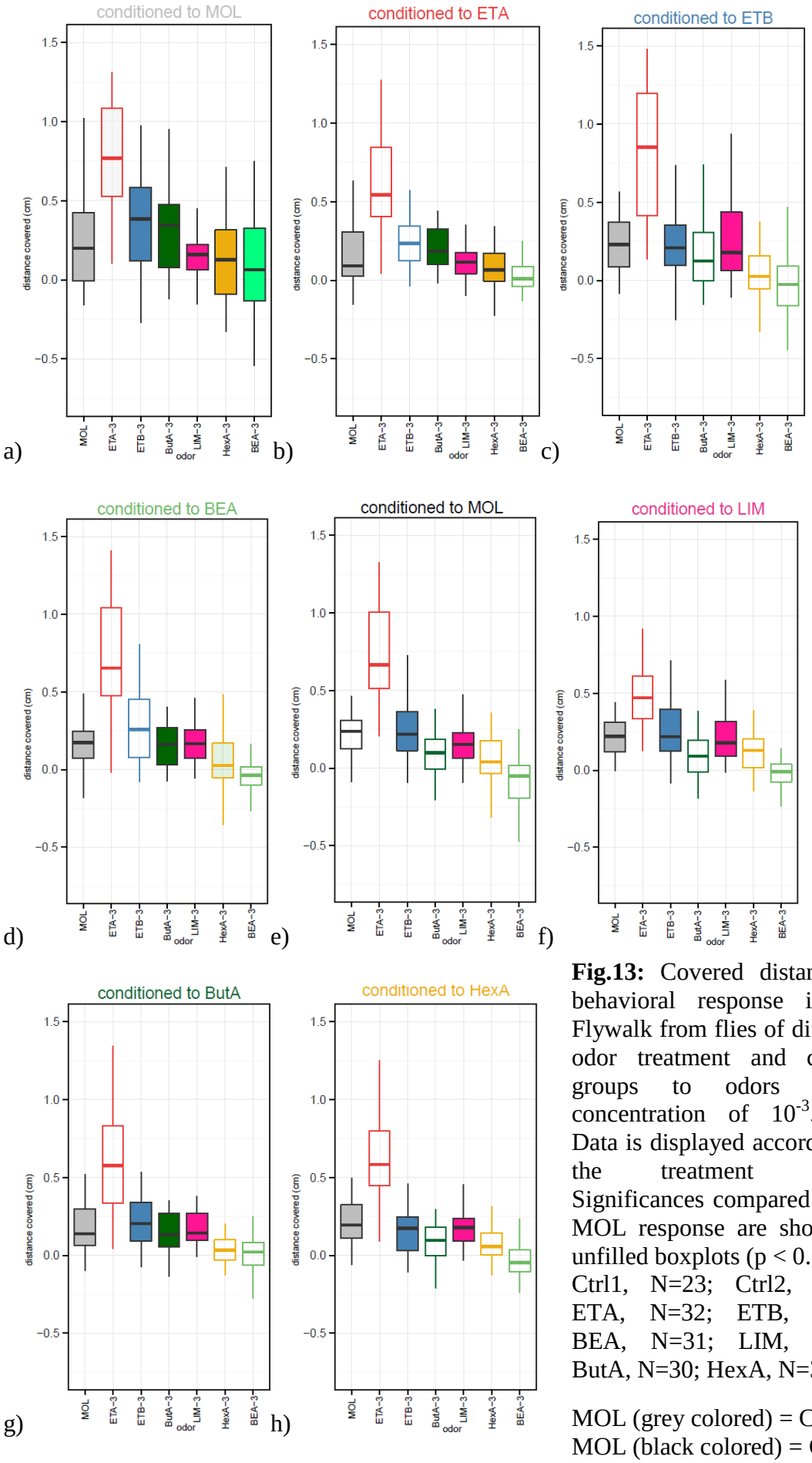


Fig.13: Covered distance as behavioral response in the Flywalk from flies of different odor treatment and control groups to odors at a concentration of 10^{-3}. The Data is displayed according to the treatment group. Significances compared to the MOL response are shown as unfilled boxplots ($p < 0.05$). Ctrl1, N=23; Ctrl2, N=30; ETA, N=32; ETB, N=32; BEA, N=31; LIM, N=30; ButA, N=30; HexA, N=30.

MOL (grey colored) = Ctrl1
MOL (black colored) = Ctrl2

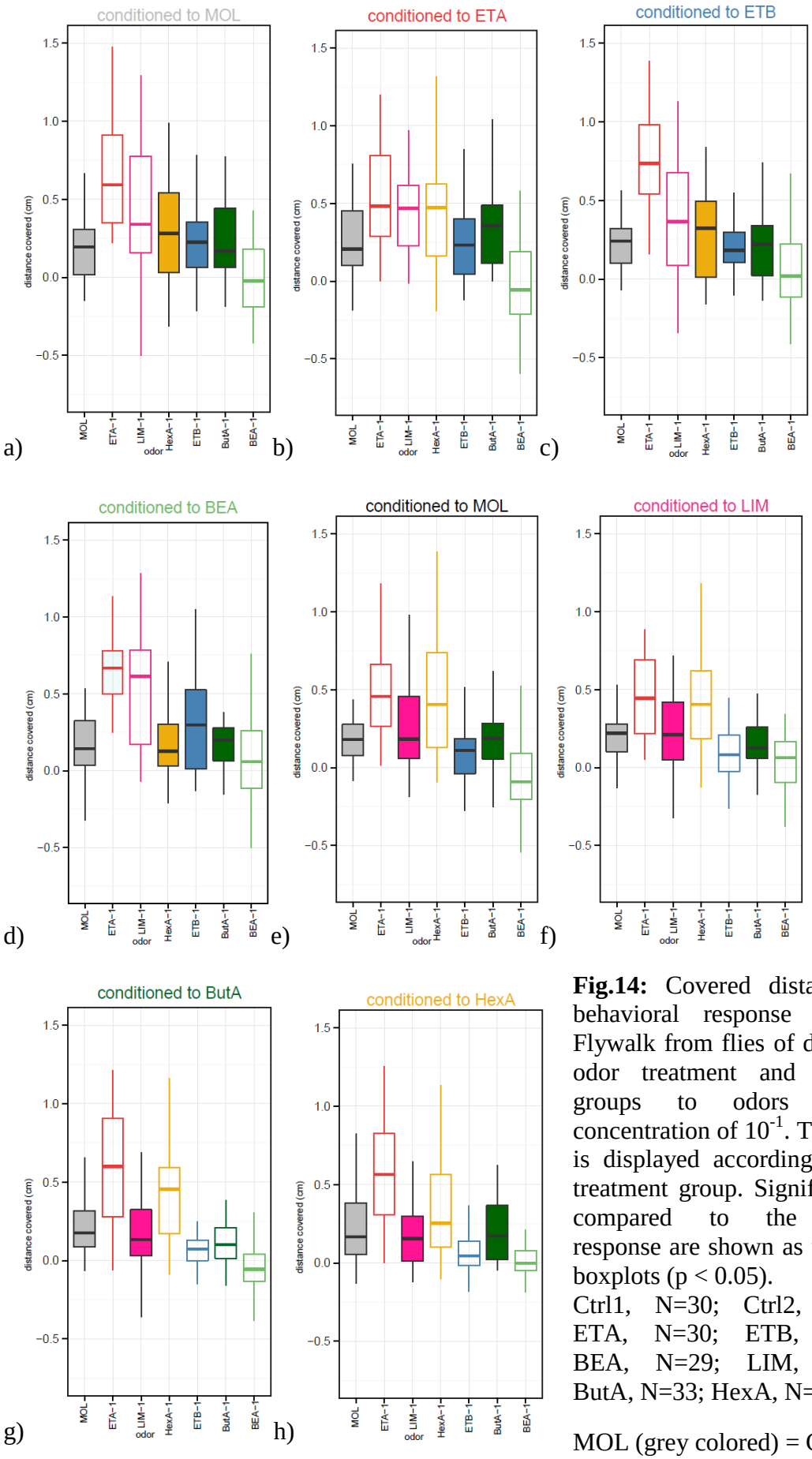


Fig.14: Covered distance as behavioral response in the Flywalk from flies of different odor treatment and control groups to odors at a concentration of 10^{-1}. The Data is displayed according to the treatment group. Significances compared to the MOL response are shown as unfilled boxplots ($p < 0.05$).
Ctrl1, N=30; Ctrl2, N=34; ETA, N=30; ETB, N=30; BEA, N=29; LIM, N=34; ButA, N=33; HexA, N=34.

MOL (grey colored) = Ctrl1
MOL (black colored) = Ctrl2

A.3 Mappings, alignments and blasts of sequencing results

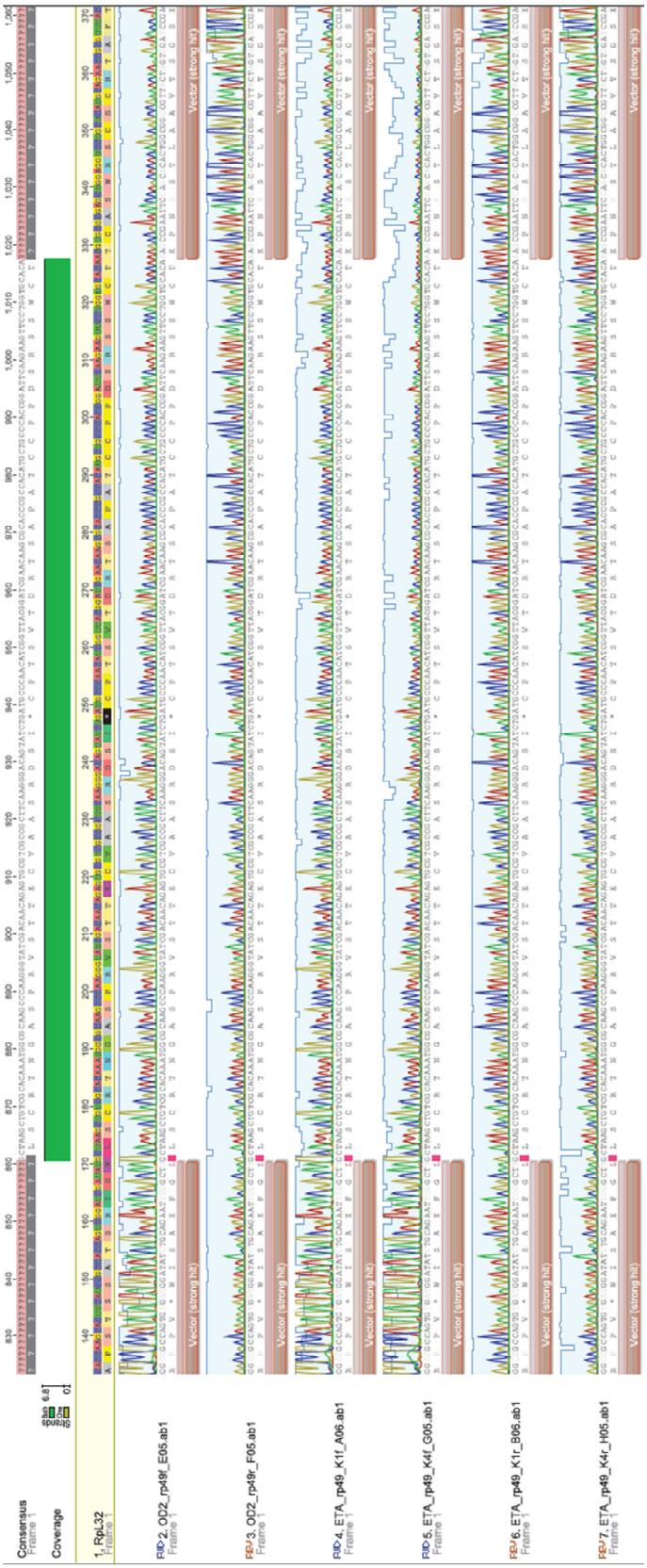


Fig.15: Mapping of the sequencing results of the housekeeping gene *rp49*, by using forward or reverse *rp49* primer, with the reference *rp49 Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

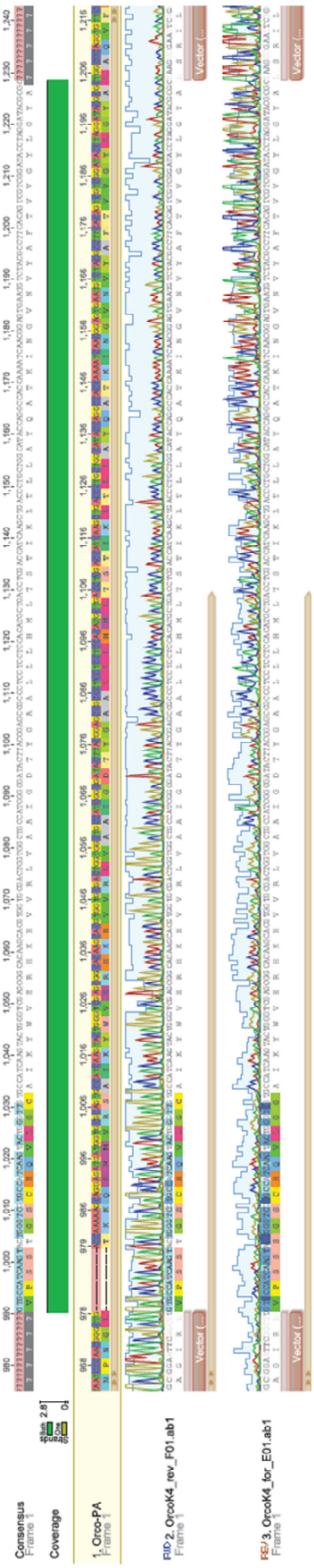


Fig.16: Mapping of the sequencing results of the co-receptor Orco, by using forward or reverse Orco primer, with the reference Orco *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

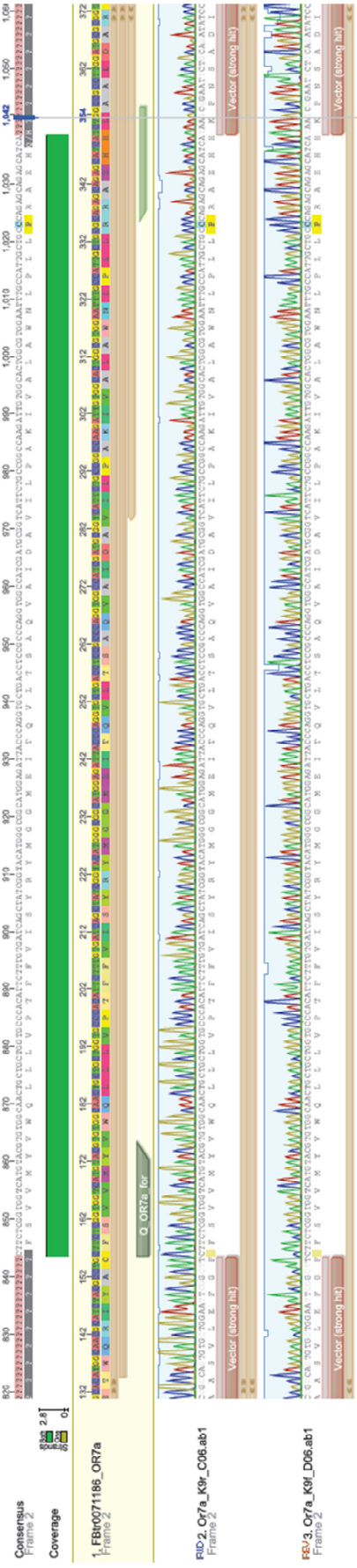


Fig.17: Mapping of the sequencing results of Or7a, by using forward or reverse Or7a primer, with the reference Or7a *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

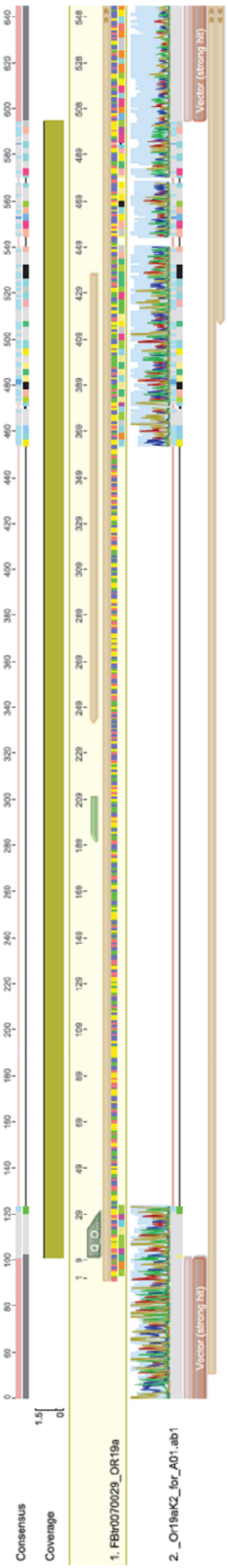


Fig.18: Mapping of the sequencing results of Or19a, by using the forward Or19a primer (first primer pair), with the reference Or19a *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

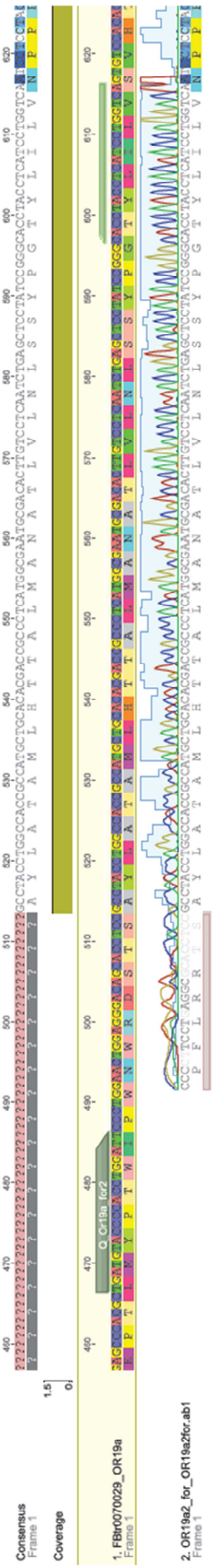


Fig.19: Mapping of the sequencing results of Or19a, by using the forward Or19a primer (second primer pair), with the reference Or19a *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

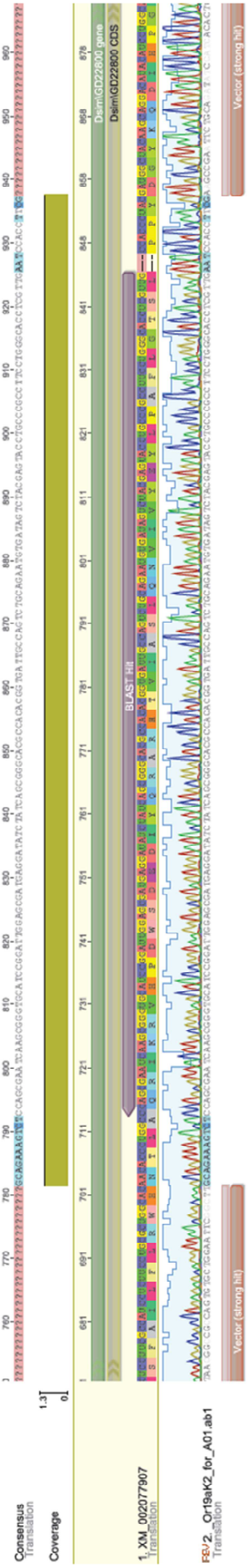


Fig.20: BLAST result of the sequencing result of Or19a, by using forward Or19a primer (first primer pair). The used program was Geneious 6.0.5. As matching sequence the gene Dsim/GD22800 of *Drosophila simulans*, a Dual oxidase, was obtained.

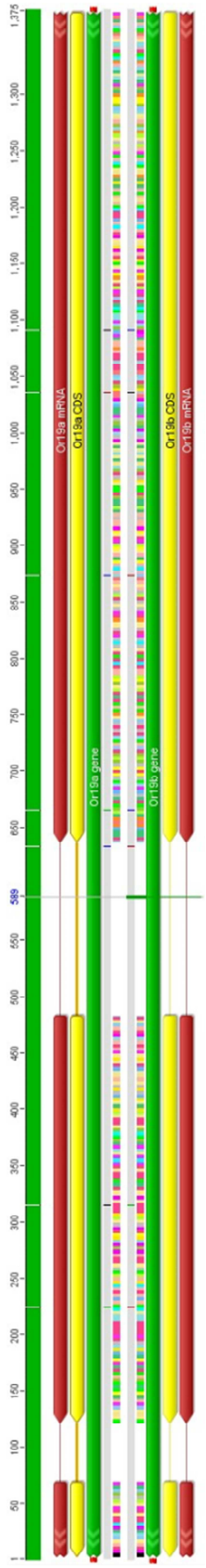
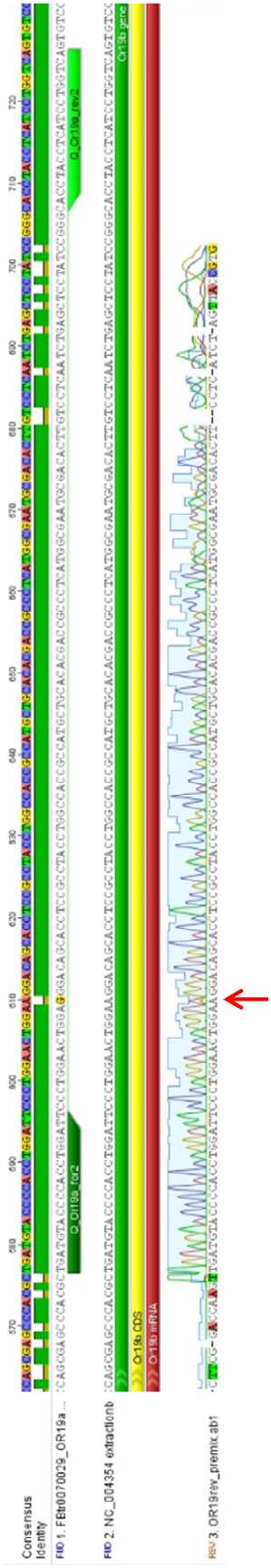


Fig.21: Alignment of Or19a and Or19b reference sequences. The sequences of both Ors are nearly identical. They differ only in 6 bp, distributed over the sequence. Both coding sequences have 3 exons. One further bp difference is positioned in the intron sequence.



Alignment of the obtained sequence by using the reverse second Or19a primer to the reference sequences of Or19a and Or19b showed an amplification of both receptors. Both receptors have a base pair difference at position 610 in the reference sequences, with guanine in Or19a and adenine in Or19b. Both bases are present in the sequencing results at position 610 of the sequence.

Fig.22: Alignment of the sequencing results of Or19a, by using the reverse Or19a primer (second primer pair), with the reference Or19a and Or19b *Drosophila melanogaster* sequence. Both, Or19a and Or19b, Or fragments were amplified by using the second reverse Or19a primer. The reference sequences were obtained from www.flybase.org. The used program was Geneious 6.0.5.

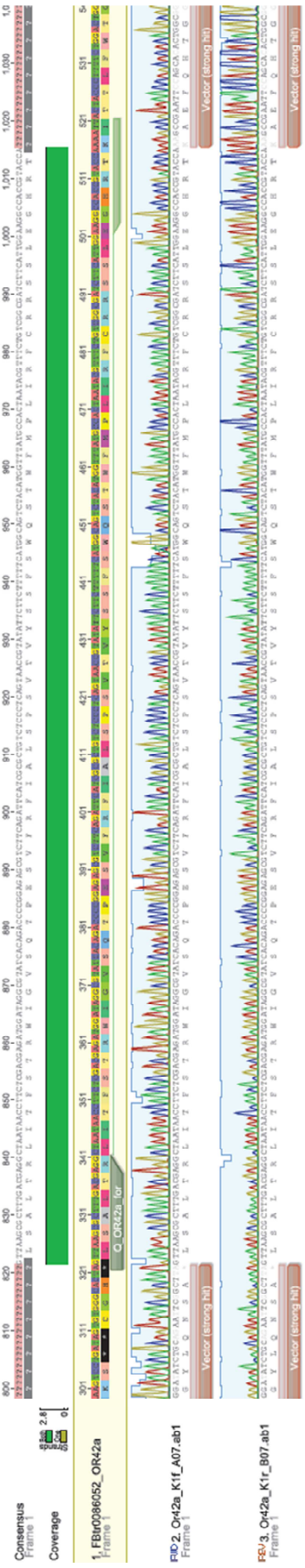


Fig.23: Mapping of the sequencing results of Or42a, by using forward or reverse Or42a primer, with the reference Or42a *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

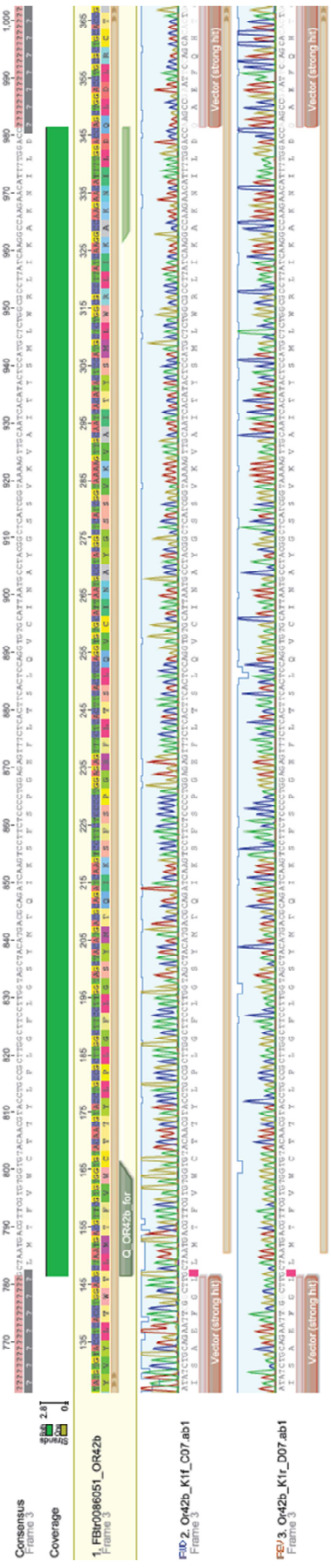


Fig.24: Mapping of the sequencing results of Or42b, by using forward or reverse Or42b primer, with the reference Or42b *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

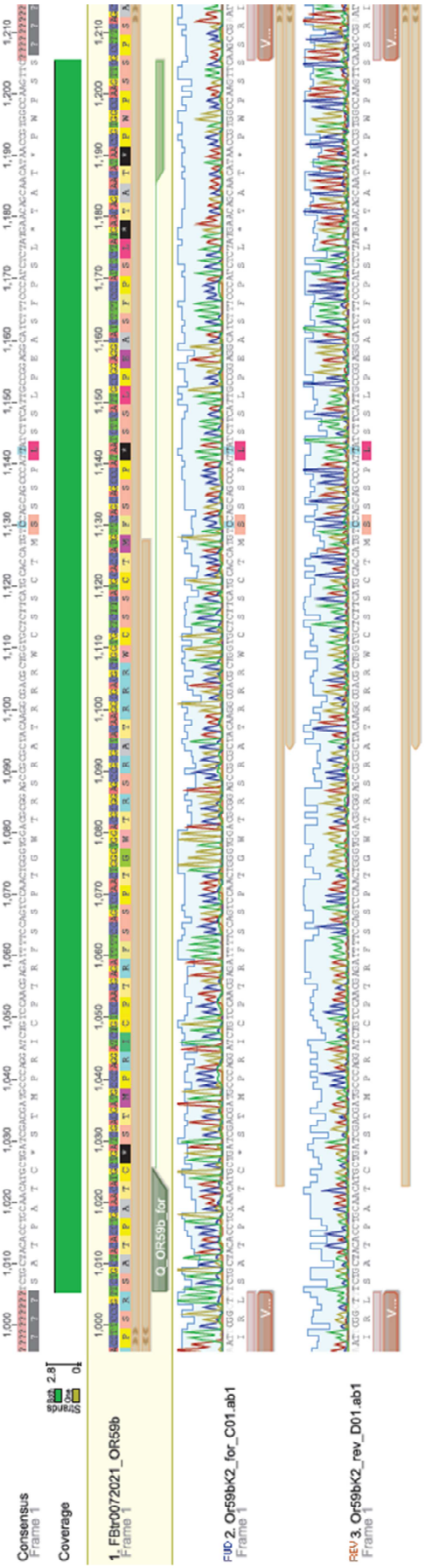


Fig.25: Mapping of the sequencing results of Or59b, by using forward or reverse Or59b primer, with the reference Or59b *Drosophila melanogaster* sequence. The reference sequence was obtained from www.flybase.org. The used program was Geneious 6.0.5.

A.4 STDEV of the technical replicates in the qPCR

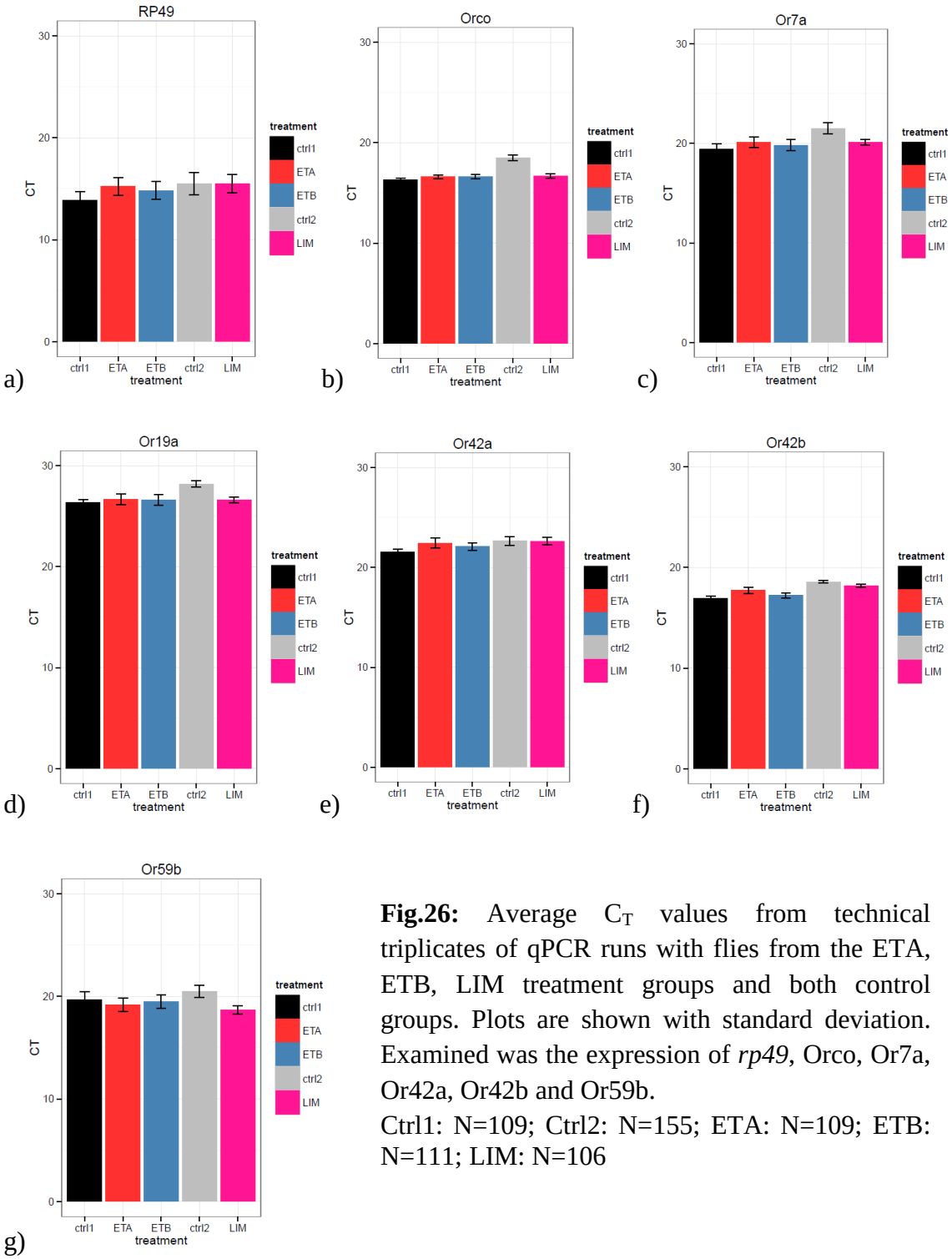


Fig.26: Average C_T values from technical triplicates of qPCR runs with flies from the ETA, ETB, LIM treatment groups and both control groups. Plots are shown with standard deviation. Examined was the expression of *rp49*, Orco, Or7a, Or42a, Or42b and Or59b.
Ctrl1: N=109; Ctrl2: N=155; ETA: N=109; ETB: N=111; LIM: N=106

A.5 Odor exposure during larval stages of *Drosophila* until pupation

a)  b) 

Fig.27: Rearing of Canton S *Drosophila melanogaster* larvae until pupation during odor exposure. a) *Drosophila* larvae exposed to benzaldehyde. The odor containing filter paper was exchanged daily. They started to pupate distributed in the whole food Vial, even directly at the odor containing filter paper. The pupae were immediately transferred to a fresh food Vial without odor. b) The *Drosophila* larvae do not avoid the odor containing filter paper (in this case: BEA-1).

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Declaration of original autorship

I hereby declare that the submitted Master Thesis is my own and that all passages and thoughts that are contributions from other sources are recognizable and clearly marked. The Master Thesis was not used to achieve an academic grading elsewhere.

Jena, 08.11.2014 ……