Classical and operant learning in the larvae of *Drosophila melanogaster*

Klassiches und operantes Lernen bei Larven der *Drosophila melanogaster*

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation Klassiches und operantes Lernen bei Larven der Drosophila melanogaster. eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
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Acknowledgements

Summary

In this thesis I studied psychological aspects in the behaviour of *Drosophila*, and especially *Drosophila* larvae. After an introduction where I present the general scientific context and describe the mechanisms of olfactory perception as well as of classical and operant conditioning, I present the different experiments that I realised during my PhD.

Perception The second chapter deals with the way adult *Drosophila* generalise between single odours and binary mixtures of odours. I found that flies perceive a mixture of two odours as equally similar to the two elements composing it; and that the intensity as well as the physico-chemical nature of the elements composing a mixture affect the degree of generalisation between this mixture and one of its elements. These findings now call for further investigation on the physiological level, using functional imaging.

Memory The third chapter presents a series of experiments in *Drosophila* larvae in order to define some characteristics of a new protocol for classical aversive learning which involves associating odours with mechanical disturbance as a punishment. The protocol and the first results should open new doors for the study of classical conditioning in *Drosophila* larvae, by allowing the comparison between two types of aversive memory (gustatory *vs.* mechanical reinforcement), including a comparison of their neurogenetic bases. It will also allow enquiries into the question whether these respective memories are specific for the kind of reinforcer used.

Agency The fourth chapter documents our attempts to establish operant memory in *Drosophila* larvae. By analysing the first moments of the test, I could reveal that the larvae modified their behaviour according to their previous operant training. However, this memory seems to be quickly extinguished during the course of the test. We now aim at repeating these results and improving the protocol, in order to be able to systematically study the mechanisms allowing and underlying operant learning in *Drosophila* larvae.

In the fifth chapter, I use the methods developed in chapter four for an analysis of larval locomotion. I determine whether larval locomotion in terms of speed or angular speed is affected by a treatment with the "cognitive enhancer" *Rhodiola rosea*, or by mutations in the *Synapsin* or *SAP47* genes which are involved in the formation of olfactory memory. I also characterize the modifications induced by the presence of gustatory stimuli in the substrate on which the larvae are crawling.

This thesis thus brings new elements to the current knowledge of *Drosophila* psychology and will hopefully open new directions of research in this particular field.

Zusammenfassung

In dieser Doktorarbeit studiere ich einige psychologische Aspekte im Verhalten der *Drosophila*, insbesondere von *Drosophila* Larven. Nach einer Einleitung, in der ich den wissenschaftlichen Kontext darstelle und die Mechanismen der olfaktorischen Wahrnehmung sowie des klassichen und operanten Lernens beschreibe, stelle ich die verschiedenen Experimente meiner Doktorarbeit vor.

Wahrnehmung Das zweite Kapitel behandelt die Art, in der adulte *Drosophila* zwischen Einzeldüften und Duftgemischen generaliseren. Ich habe gefunden, daß die Fliegen eine Mischung aus zwei Düften als gleich verschieden von ihren beiden Elementen wahrnehmen; und daß die Intensität sowie die chemisch-physikalische Natur der Elemente das Ausmass der Generalisierung zwischen der Mischung und ihren beiden Elementen beeinflusst. Diese Entdeckungen sollten für die weitere Forschung anregend sein, wie zum Beispiel zum functional imaging.

Gedächtnis Das dritte Kapitel stellt die Etablierung eines neuen Protokolls zur klassischen Konditionierung bei *Drosophila* Larven dar. Es handelt sich um Experimente, bei denen ein Duft mit einer mechanischen Störung als Strafreiz verknüpft wird. Das Protokoll wird einen Vergleich zwischen zwei Arten vom aversiven Gedächtnissen (Geschmack *vs.* mechanische Störung als Strafreize) ermöglichen, einschliesslich eines Vergleiches ihrer neurogenetischen Grundlagen; zudem kann nun geforscht werden, ob die jeweiligen Gedächtnisse spezifisch für die Art des verwendeten Strafreizes sind.

Selbstgestaltung Das vierte Kapitel umfasst unsere Versuche, operantes Gedächtnis bei *Drosophila* Larven zu beobachten. Zumindest für die unmittelbar ersten Momente des Tests konnte ich zeigen, dass die Larven ihr Verhalten entsprechend dem Training ausrichten. Dieses Gedächtnis scheint jedoch im Laufe des Tests schnell zu verschwinden. Es ist daher geraten, diese Ergebnisse über operantes Lernen zu wiederholen, eventuell das experimentelle Protokoll zu verbessern, um so eine systematische Analyse der Bedingungen und Mechanismen für das operante Lernen bei der *Drosophila* Larve zu erlauben.

Im fünften Kapitel verwende ich die im Rahmen des vierten Kapitels entwickelten Methoden für eine Analyse der Fortbewegung der Larven. Ich habe insbesondere die Wirkung des pflanzlichen ,cognitive enhancers' *Rhodiola rosea* untersucht, sowie die Auswirkungen von Mutationen in den Genen, welche für *Synapsin* und *SAP47* kodieren; schliesslich habe ich getestet, ob die Geschmacksqualität der Testsituation lokomotorische Parameter verändert.

Diese Dissertation erbringt also eine Reihe neuer Aspekte zur Psychologie der *Drosophila* und wird hoffentlich in diesem Bereich der Forschung neue Wege öffnen.

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Chapter 1.

Introduction

1.1 Preface: learning and cognition

> *"Intelligence is what you use when you don't know what to do."* Jean Piaget

The study of behaviour as the observable result of brain activity started early in the history of natural sciences. Darwin already tried to deduce the thoughts and emotions from animal behaviour and to compare them to humans (Darwin 1871 and 1872). The systematic methodology for this approach, that is noting behavioural changes following a training phase, is due to experimental psychology initiated in beginning XXth century by Pavlov (1927), Skinner (1938) and Thorndike (1898). They introduced the first laws that are governing learning and memory, such as stimulus strength or temporal pairing. In the second half of the XXth century, cognitive psychologists, such as Kamin (1969) with the blocking phenomenon, showed that pairing between stimuli is not always sufficient to induce learning and that higher order processes like attention, surprise and predictability also take place.

The phenomenon of associative conditioning is studied in many disciplines, from psychology, education, to neurology, neurobiology and artificial intelligence. It allows extracting causal rules existing in the surroundings of an individual. It thus relies strongly on the venue, the timing, the nature and the strength of external stimulations, as it has been precociously formalised by early experimental psychology and behaviourism. However, in a constantly changing environment, associative conditioning cannot be a fully fixed and automatised process but has to be supervised by higher internal brain functions, in order for example to generalise between recognizably different stimuli, or to pre-emptively act in the venue of stimuli through expectations. Such cognitive processes 'infest' most if not all non reflexive behaviours in vertebrates as well as invertebrates (*e.g.* in insects: Stevenson *et al*. 2005, Wystrach *et al*. 2011, Stach *et al*. 2004, Avarguès-Weber *et al*. 2010). *Drosophila melanogaster*, in its adult or larval form, has taken an important place in the study of these

questions as it allows studying the genetic bases of any biological function (*e.g.* Neuser *et al*. 2008, Colomb and Brembs 2010, Pauls *et al*. 2010b, Ofstad *et al*. 2011, Michels *et al*. 2011).

In my thesis some psychological processes in *Drosophila melanogaster* will in particular be under focus. First, I will tackle a specific question on olfaction in *Drosophila*, namely the way monomolecular odours or binary odour mixtures are perceived and generalised (Chapter 2). The degree of generalisation depends mainly on the judgement of similarity between the two odours and on the advantages conferred by ignoring recognizable differences in a given context (Mishra *et al*. 2010). The material of this chapter is published in in Chemical Senses (Eschbach *et al*., 2011a). The following chapters will consider larval *Drosophila* as a study case. Its brain – in term of cell number rather than cell organisation – and behaviour are less complex than adult flies, which makes it as a very interesting model system in neuroscience (Gerber and Stocker 2007). In larvae as well, behaviour is governed by a centralised brain, and information processing takes place. Using a new kind of negative reinforcement of unpleasant mechanical disturbance, I will present experiments designed to investigate classical conditioning abilities in larvae (Chapter 3), including the question which kinds of threat can motivate the larvae for expressing conditioned escape. The material of this chapter is published in The Journal of Experimental Biology (Eschbach *et al*., 2011b). In Chapter 4, I will explore the operant conditioning abilities of the larvae. Last, I will examine which environmental factors can influence larval locomotion (Chapter 5). Taken together, these experiments hopefully bring some cues on how behaviour in *Drosophila* is related to environmental conditions, which internal processes contribute to this relation.

1.2 Cognition in *Drosophila*

1.2.1 Generalities about *Drosophila melanogaster*

Drosophila melanogaster, commonly named as *Drosophila*, is a species of the family of Drosophilidae of the dipterian insects. After its introduction as a laboratory animal by Castle at Harvard University in 1901 it became one of the most used model organisms in biological studies due to its small size allowing large-scale manipulations, the relatively little care it requires, its high fecundity and short generation cycle, and the many experimental opportunities it thus offers to study rare events in genetic studies. Among them, genetic transformation techniques available and the invention of the Gal4/UAS system, a powerful

genetic "Swiss-army knife" (Brand and Perrimon, 1993) opened many doors also in neurobiological research. Combined with experimental psychology, it led to a detailed picture of some aspects of behavioural plasticity in *Drosophila* (reviewed in McGuire *et al*. 2005). Furthermore, genetic mechanisms are mostly shared across eukaryotes and the discovery of some genetic processes in fruitflies can be generalised to a certain extent to other species, including humans (Adams *et al*. 2000).

The life of a fruitfly in laboratory conditions, that is, in mass and at 25°C, can be described in a few sentences. Larval hatching occurs one day after egg-laying and larvae grow for the following four days, molting twice into second-instar at 24 h and third-instar larvae at 48 h. During this larval stage, they feed the decomposing fruits on which they are living. At the end of the 5th day, they undergo a four-day long pupal quiescence; after emergence from the pupal case, flies become soon mature so that the total generation cycle lasts for around 10 days.

Accompanying body transformation, the nervous system is mostly reorganised during metamorphosis with reuse, re-specification or apoptosis of larval neurons together with the generation of new specific adult neurons from the imaginal discs. Almost all adult motoneurons are remodelled embryonic-born motoneurons whose target muscles are redefined between larval and adult stages (Tissot and Stocker 2000). Concerning the sensory neurons, most of the gustatory neurons are embryonic-born, and persist from larval to adult stage although the adult sensory organs are more complex and include adult-specific neurons as well (Gendre *et al*. 2004, Ramakaers *et al* 2005). The other sensory neurons seem to mostly die during metamorphosis and new neurons are generated *de novo* from the imaginal disc. Interneurons in the adult central nervous system derive either from embryonic-born interneurons or are larval-born and are added during larval or pupal stages (Tissot and Stocker 2000). The adult is thus a mosaic of embryonic-born neurons which persisted, and larval-born neurons which are incorporated into adult system during pupal stages.

Adult flies exhibit many behaviours subject to plasticity in social (Griffith and Ejima 2009, Dahanukar and Ray 2011) or individual new contexts (*e.g.* getting over obstacles: Pick and Strauss 2005, complex pattern recognition: Dill *et al*. 1993, Liu *et al*. 1999), as well as learning and memory abilities (Tully & Quinn 1985, Wolf and Heisenberg 1991, Zars 2009, Neuser *et al*. 2008). The behaviour of *Drosophila* larvae, with less dimensions, proved to

involve some plasticity (*e.g.* Ruiz-Dubreuil *et al.* 1996, Kaun *et al.* 2007, Mishra *et al.* 2010) as well as simple associative learning (Scherer *et al.* 2003, Yarali *et al.* 2006, Gerber and Hendel 2006).

To summarize, fruitflies are valuable for studying how a small animal with a few neurons developed to face its environment (Heisenberg 1997). Larvae, with a numerically yet simpler brain, a simpler body, and a simpler behavioural repertoire (Gerber and Stocker 2007, Gerber *et al.* 2009) also represent a very interesting alternative model system in neuroscience. Considering in particular the larvae, their cognitive limits are not yet clear; with this thesis I would like to contribute to finding these limits. In the following part of the introduction, I will give a short overview on the knowledge accumulated on some specific cognitive questions in *Drosophila*, adult or larva.

1.2.2 Perception: olfaction

> "*Zehntausend, hunderttausend spezifische Eigengerüche hatte er gesammelt und hielt sie zu seiner Verfügung, so deutlich, so beliebig, daß er sich nicht nur ihrer erinnerte, wenn er sie wiederroch, sondern daß er sie tatsächlich roch, wenn er sich ihrer wiedererinnerte.*" Patrick Süskind, Das Parfum

The way sensory inputs are organised in order to extract meaningful information is important for the survival of the animal. Olfaction is a major perceptual sense in *Drosophila*, with major roles in many aspects of their life as it signals them location of conspecifics, food, egg laying sites or danger (*e.g.* Mery and Kawecki 2002, Siwicki *et al.* 2005, Ejima *et al.* 2005, McBride *et al.* 2007). The determination of an odour quality is ensured thanks to the design of the olfactory system, where different odours can cause different neuronal activity patterns along the olfactory pathway (reviewed in Stocker 1994, Strausfeld and Hildebrand 1999, Galizia and Menzel 2000, Hallem *et al.* 2006, Vosshall and Stocker 2007, Gerber *et al.* 2009, Masse *et al.* 2009).

In adult *Drosophila*, 62 types of olfactory receptors (OR) of the *Or* family have been identified that ensure olfactory transduction (Clyne *et al.* 1999, Vosshall *et al.* 1999, see also Benton *et al.* 2009) and that are tuned to specific classes of ligands (Clyne *et al.* 1999, Vosshall *et al.* 1999). Each olfactory sensory neuron expresses only one type of OR – together

with the ubiquitous co-receptor *Orco* (formerly *Or83b*, Larsson *et al.* 2004, renamed by Vosshall and Hanson 2011) –, so that its response properties are defined by its specific OR. These neurons, located on the maxillary palp as well as the antenna, convey the information along the maxillary and antennal nerves to the antennal lobe, the primary olfactory centre in insects, where they are regrouped in glomeruli according to the receptor they express (Fig 1.1). These glomeruli have been shown to be functional units: in vivo calcium imaging approaches revealed that the application of an odour specifically and stereotypically activates a combination of those glomeruli (Galizia *et al.* 1999, Sachse *et al.* 1999). Also, chemically similar odours activate similar activity patterns (Guerrieri *et al.* 2005). In each glomerulus, modifications of the signal occur through connections between the afferent olfactory receptor neurons, local inhibitory or excitatory interneurons, and the efferent projection neurons. The projection patterns of the interneurons range from "glomerulus-glomerulus specific" to "all glomeruli" in the antennal lobe (Chou *et al.* 2010, Huang *et al.* 2010, Yaksi and Wilson 2010). Further in the circuit, projection neurons convey the reshaped signal directly to the lateral horn, as well as indirectly through a detour via the mushroom bodies. This detour seems to be dedicated to the organisation of learnt behaviour as the Kenyon cells, intrinsic neurons of the mushroom bodies, show molecular plasticity leading to the formation of associative olfactory memory (Heisenberg 2003, Gerber *et al.* 2004b, Krashes *et al.* 2007, Masse *et al.* 2009). The direct lateral horn circuit seems relatively more hard-wired and is sufficient for innate olfactory responses such as the courtship behaviour (Heimbeck *et al.* 2001, Cachero *et al.* 2010).

Interestingly, the global organisation of the whole circuit is tightly conserved among the insects (review by Galizia and Rössler 2010), and is similar to the olfactory system of mammals as well, where first-order receptor neurons expressing one type of olfactory receptor converge in glomeruli at the olfactory bulb and form synapse with the mitral cells that project further in cortical areas dedicated to olfactory processing (Bargmann 2006).

The organisation of the olfactory system, and especially the various connectivities of the interneurons, is thought to improve the signal-to-noise ratio and the separability between odour qualities (Fdez Galán *et al.* 2004, Linster *et al.* 2005, Silbering *et al.* 2008). However, the bases on which this quality is defined, as for example physico-chemical or biologically relevant characteristics, are not yet well understood (Schmuker and Schneider 2007, Haddad *et al.* 2008).
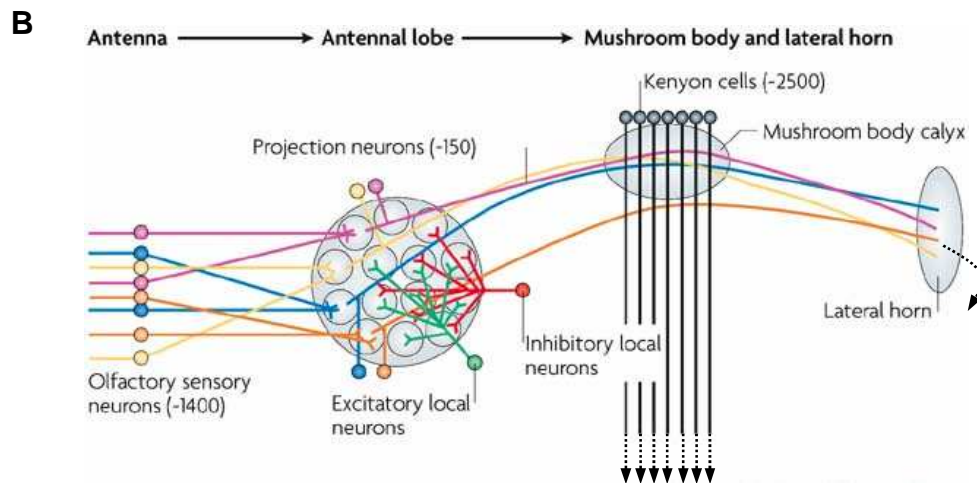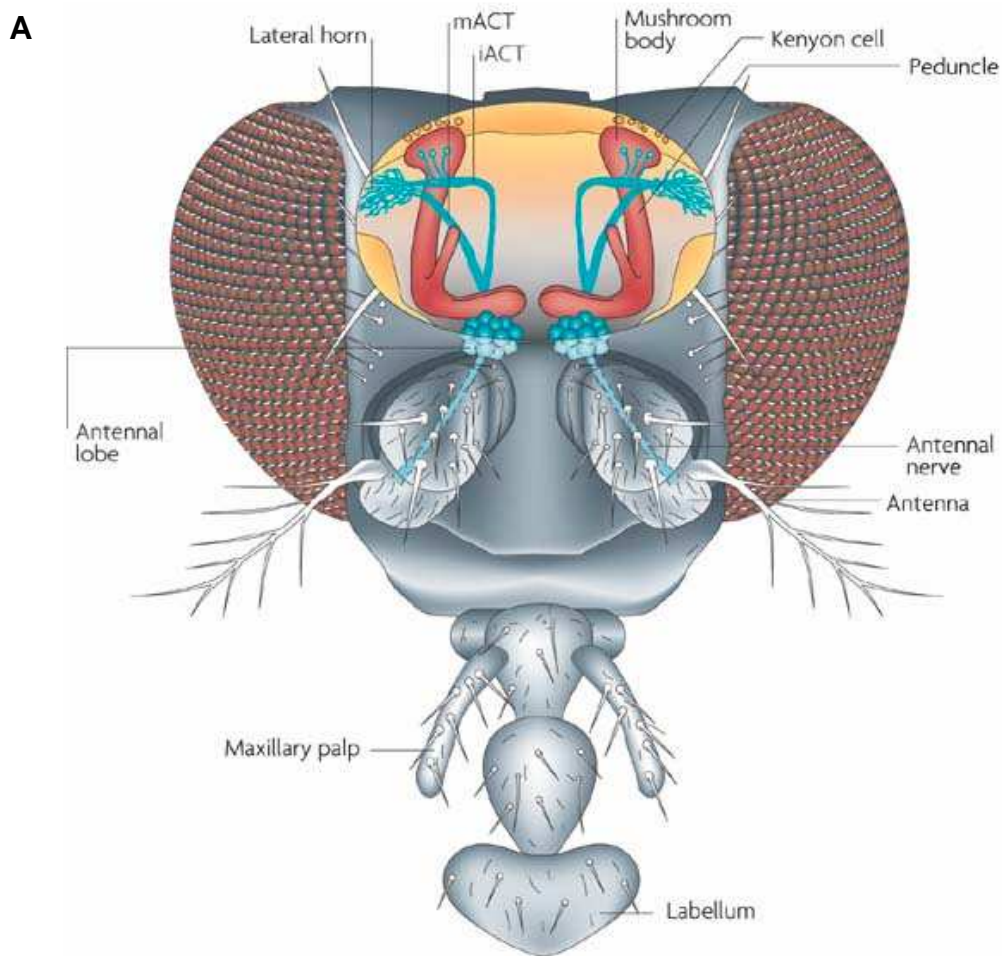
Figure 1.1. Olfactory system in *Drosophila melanogaster*: sketch of the anatomy (A) and of the connections (B). Olfactory sensory neurons detect the odours presented at the antennae, then project towards the antennal lobe, where they regroup in glomeruli according to the receptor type they express. Synaptic connections are made with local interneurons and projection neurons. These latter ones convey then the signal further towards the Kenyon cells of the mushroom bodies and the cells of the lateral horn. Note that the projections of olfactory sensory neurons from the maxillary palp towards the antennal lobe are omitted. The figures are modified from Keene and Waddell, Nature Reviews Neuroscience 2007.

The study of odours presenting high similarity is of interest to understand how the system manages fine discrimination. In particular, mixtures of odours convey an ambiguous message, resembling its components although still being clearly discriminated from it by the flies (see Chapter 2). The way the olfactory system processes these kinds of information starts to be unravelled: Comparison between activity patterns evoked by a mixture and its components at the input (receptor neurons) and output (projection neurons) of the antennal lobe revealed that a mixture signal is the sum of its component signals at the input, while it is different from that sum at the output of the antennal lobe (Silbering and Galizia 2007, Deisig *et al*. 2006 and 2010). The resulting signal is thus being better differentiated after computation in the antennal lobe (Deisig *et al*. 2006 and 2010), certainly helping odour discrimination.

In Chapter 2, I adress how odour mixtures and components are treated by the flies, and for that I "asked" flies how much of a single odour they would recognise in a mixture of two, or in turn how much of a mixture of two odours they would recognise in only one component of it. These behavioural experiments followed the below-design:

Training (pairing with electric shock)	Test (avoidance of the odour)
A	AB
AB	A

The logic behind these experiments is that the more flies perceptually confound the trained and the tested stimulus, the more the conditioned response will be as strong as if the trained and tested odour were actually the same (Pavlov 1927, Guerrieri *et al*. 2005). The description and interpretation of the results obtained can be found in Chapter 2.

1.2.3 Associative learning

Two basic forms of associative learning are known, which allow detecting, remembering and taking advantages of the causal links that exist in the world of an animal. Classical and operant conditioning are thought to be complementary processes that organise spontaneous behaviour (see Box 1.2).

Classical conditioning

Classical conditioning (Pavlov 1927) allows an animal making predictions about stimuli in its environment. Formally, a first "conditioned" stimulus (CS) is followed by the presentation of a second "unconditioned" stimulus (US) having a strong biological value for the animal, either appetitive or aversive. From this temporal pairing, the first stimulus acquires a predictive value over the venue of the second one, and can then by itself evoke an anticipatory behavioural manifestation. What is behind this observed response is an important issue in the psychological and neurobiological point of view.

According to Rescorla and Wagner (1972) the temporal pairing between CS and US results in the formation of expectations about the venue of the US when the CS is presented. In monkeys, dopaminergic neurons signal for the expected reward value of a CS (reviewed in Schultz 2002). In adult *Drosophila* dopamine serves to mediate and predict aversive stimuli (Riemensperger *et al.* 2005). Such expectations are also strongly suspected in *Drosophila* larvae (Gerber and Hendel 2006, and see box 1.1 and Chapter 3). The existence of such anticipatory abilities discredits a vision of classical conditioning as automatic process where the CS becomes directly associated with the behavioural response normally triggered by the US. However it should be noted that an intense training leads to the formation of habits in *Drosophila*, where the fly responds automatically to the CS whichever are its consequences (Brembs 2009a).

In neurobiological terms, learning is mediated by cellular modifications occuring at the convergence between the pathways of predictive and reinforcing stimuli. Taking the case of olfactory learning in adult *Drosophila*, the olfactory signal, carried by the projection neurons, and the appetitive or aversive signal, mediated respectively by octopaminergic and dopaminergic neurons likely ascending from the gustatory neurons (Schwaerzel *et al.* 2003), converge at the level of the Kenyon cells in the mushroom bodies (reviewed in Gerber *et al.* 2004b, Krashes *et al.* 2007). Our current knowledge of the consequent cellular and molecular modifications imply the *rutabaga* type I Adenylyl Cyclase as coincidence detector of the two stimuli (Tully and Quinn 1985, Dudai *et al.* 1985, Zars *et al.* 2000, Schwaerzel *et al.* 2003), and a resulting activation of the cAMP/PKA cascade (Tomchik and Davis 2009, Gervasi *et al.* 2010) responsible for memory trace formation (Fig. 1.2). In *Drosophila* larvae, reinforcements used for olfactory learning are either gustatory (Scherer *et al.* 2003), or

electric shock (Pauls *et al*. 2010a). In the case of olfactory conditioning with gustatory reinforcement, learning is supported by comparable neural pathways (Gerber and Stocker 2007, Schroll *et al*. 2006, Selcho *et al*. 2009) and seems to be supported by the same mechanisms in mushroom bodies cells as in adult (Michels *et al*. 2005, Pauls *et al*. 2010b, Michels *et al*. 2011). The training procedure however does not allow temporal manipulation of the reinforcement as the larvae are directly crawling on the tasting substrates. In the case of olfactory conditioning with electric shock, so far the pathways involved in "electric sensation" have defied discovery. For those reasons we were looking for a new type of reinforcement, which could be easily controlled and whom sensory processing are well understood.

In Chapter 3, I therefore introduce a new olfactory conditioning protocol which used a computer-controlled loudspeaker to deliver aversive reinforcement by vibration of the substrate on which the larvae crawled. This stimulation is unpleasant for the larvae (Chapter 3, Wu *et al*. 2011) and likely involves the tactile and proprioceptive system (reviewed in Kernan 2007). It should thus be possible to define the connectivity between CS and US pathways and define the cellular mechanisms involved in this type of aversive memory, in particular regarding US processing. Our reinforcement may also present the advantage to be ecologically more relevant than electirc shock, as it resembles the buzz of natural predators of the larva (Dorn *et al*. 1997, Djemai *et al*. 2001). We implemented classical conditioning by pairing an odour A with a series of vibrations while a second odour B was presented without such stimulation, and then examined the behaviour of the larvae presented with both these odours. Importantly we always used a reciprocal paradigm for the estimation of larval learning performances, that is, we trained a second group of larvae by pairing the odour B with the buzz while the odour A was presented alone. With this procedure, we ensure that the avoidance observed from the paired odour is really due to the conditioned aversion resulting from pairing with the buzz and not to any non-associative effect such as sensitisation (Préat 1998, Tully and Quinn 1985).
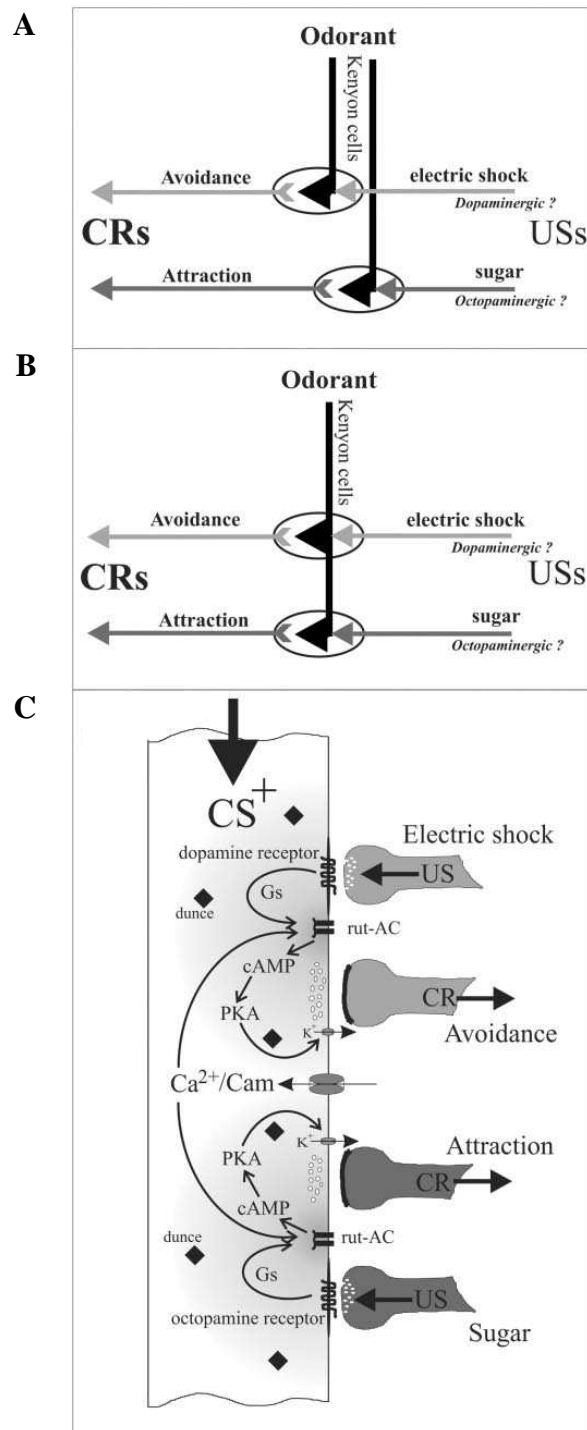
Figure 1.2. Alternative representations of olfactory memory traces. The Kenyon cells of the mushroom bodies convey information about a specific odour, which in olfactory conditioning plays the role of the CS. They receive synaptic input from modulatory neurons mediating the reinforcing properties of the US: dopaminergic neurons represent a net-aversive signal (*e.g.* electric shock) octopaminergic a net-attractive signal (*e.g.* sugar). A mushroom body output neuron mediating the conditioned response (CR neuron) is associated to the modulatory neuron and will be recruited to respond a particular odorant if the US and the odorant coincide. Depending on the way an odour is represented in the Kenyon cells, that is the degree of redundancy in the representation, a single Kenyon cell might support aversive and appetitive memory by itself (A) or either one or the other type of memory (B). C) The memory traces supporting aversive and appetitive memories would be stored in the Kenyon cells using the same molecular mechanisms, if these mechanisms are enforced in independent compartment of the Kenyon cell. From Schwaerzel, Monastirioti, Scholz, Friggi-Grelin, Birman and Heisenberg, Journal of Neuroscience 2003.

Box 1.1. Larvae and expectations

First experiment from Hendel et al. 2005:

- Groups of larvae were trained that a first odour but not a second one was paired with sugar (or bitter); a reciprocal group received the reversed contingency.

- The groups were then tested for their preference between the two odours.

- The groups trained with sugar showed conditioned attraction towards the previously rewarded odour.

- By contrast, the groups trained with bitter did not show conditioned aversion from the previously punished odour.
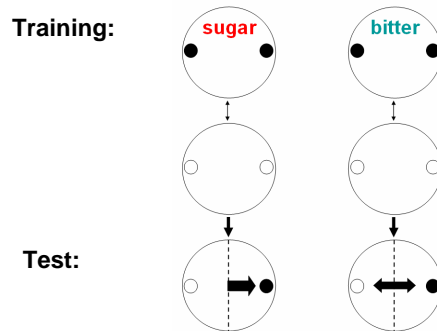
- The authors concluded from these observations that sugar and other appetitive substances tested could support associative learning whereas bitter and other aversive substances tested could not ("The carrot, not the stick"). But is it really that larvae are only able of appetitive learning and not aversive?

Second experiment from Gerber and Hendel 2006:

- The authors trained groups of larvae in the same way as the above-described experiment, using either sugar or bitter as reinforcement.

- The test however was performed in the presence of the reinforcement used for training.

- The groups trained with sugar and tested @ sugar showed no preference for the previously rewarded odour.

- The groups trained with bitter and tested @ bitter showed conditioned aversion from the odour previously punished.
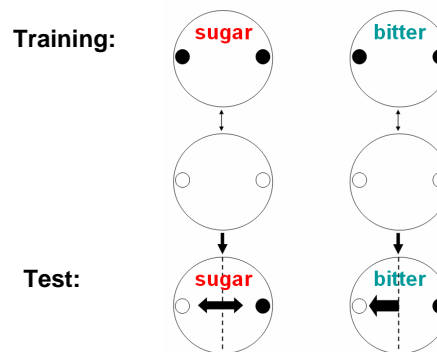
- Thus, conditioned behaviour is not an automated process. Rather, after appetitive training conditioned behaviour may better be viewed as search for the reward, whereas after aversive training, the larvae use their memory to escape the bitterness. Such conditioned search is disabled if the sought-for reward is already there, whereas in turn conditioned escape remains suppressed as long as the testing situation is agreable. In this sense, it is the expected outcome which determines whether memory is behaviourally expressed- or not. Please see Schleyer *et al* (2011) for more detail, as well as for the observation that innate olfactory behaviour is unaffected by the presence of tastants.

Operant conditioning

Operant conditioning is the second basic type of associative learning (Skinner 1938) and deals with a possible link between a "self"-initiated action and the environment. More precisely, it is learning that a particular behaviour entails particular consequences. As a result, the animal can increase or decrease the frequency of this behaviour to control the venue of the consequence. The model proposed by Wolf and Heisenberg (1991) considers several processes that are necessary for operant conditioning: activeness, efference copy of a behaviour, sensory feedback, and temporal comparison (Fig. 1.3).
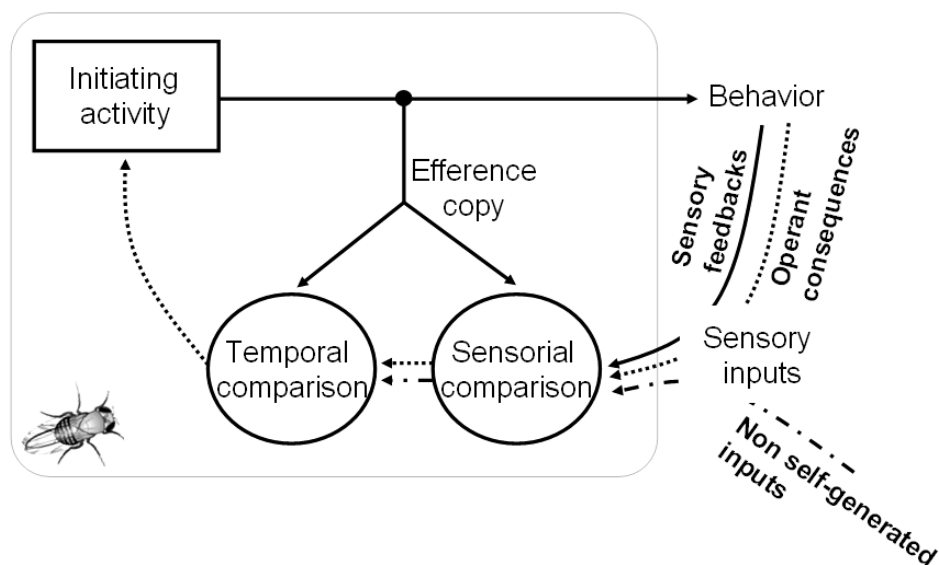
Figure 1.3. The principles of operant learning. By initiating a particular action, the animal might modify its environment or state. The consequence of this action is extracted by a sensorial comparison which substracts the current sensory inputs by the expected inputs (sensory feedbacks). The remaining signal is then temporally compared to the efference copy. When the causal link is detected, the conditioning circuit is reinforced and the propensity of the animal to initiate this action again is modified. Modified from a figure of Wolf and Heisenberg, J Comp Phys A 1991.

Activeness: Obviously, initiating activity is the starting point for this kind of learning process: only when the animal tries out and initiates different behaviours it has the possibility to find out their consequences (von Holst and Mittelstädt 1950, Brembs 2009b). In the case of "pure" operant conditioning, animals might behave in absence of any noticeable external input. Possibly, this situation of "no relevant information" is information by itself, and would trigger active search for new information. In adult *Drosophila*, the variation of behaviour in absence of external stimulation does not follow a linear model, which indicates that it is not mainly due to blank noise as depicted in Figure 1.4A (Maye *et al*. 2007). Rather some

stochastic laws seem to govern generation of behaviour (Reynolds and Frye 2007, Maye *et al*. 2007, but see Edwards *et al*. 2007), which seems to be organised by a central 'initiator' (Fig. 1.4B) when external inputs do not bring decisive information. Interestingly, bursts of uncontrolled motor activity have been recorded in *Drosophila* embryos before sensory neurons are mature (Crisp *et al*. 2008), indicating that central motor generators, the neural networks responsible for activity, can be active without sensory stimulation. However, upon maturation of the sensory system self-induced sensory inputs feed back to organise further action. Indeed, disrupting proprioceptive feedback disorganises locomotion in *Drosophila* larvae (Suster and Bate 2002, Caldwell *et al*. 2003, Ainsley *et al*. 2003, Song *et al*. 2007,
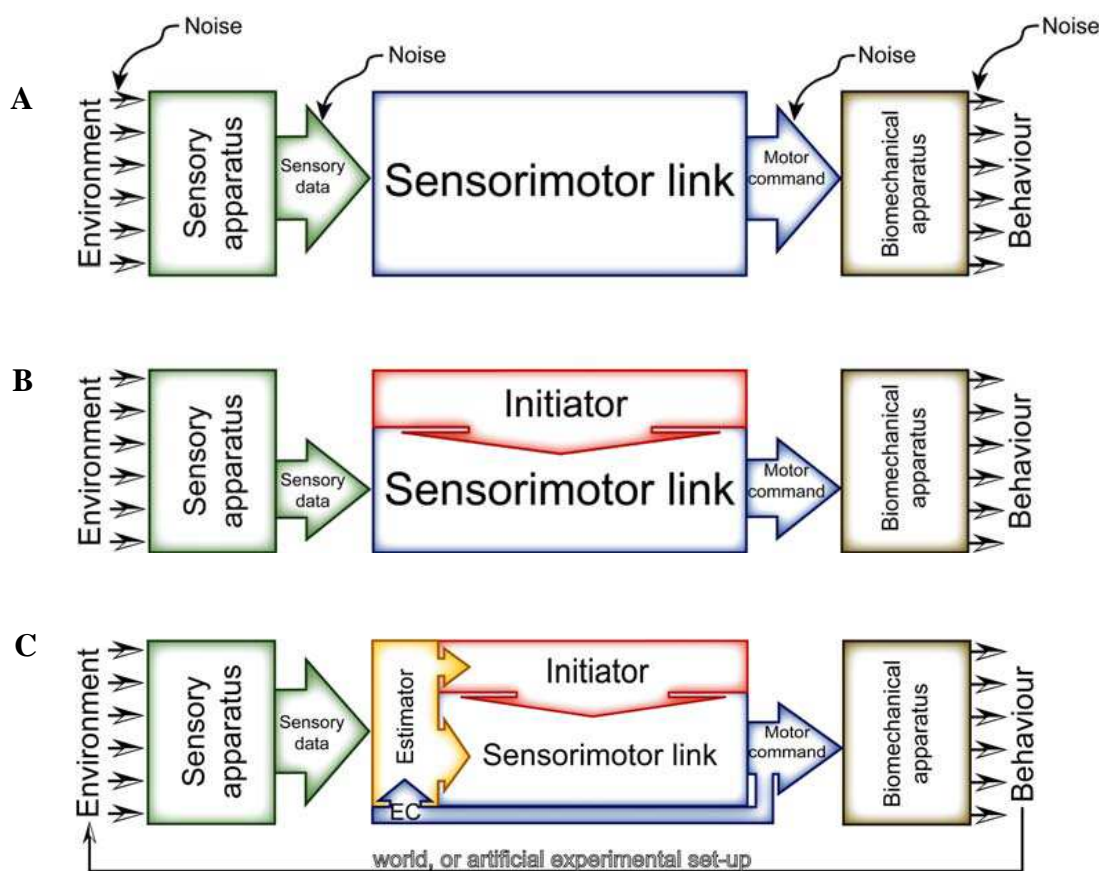
Figure 1.4. Alternative models conceptualizing "spontaneous behaviour" in *Drosophila*. A) According to the robot-hypothesis, the link between sensory input to behavioural output is unambiguous, if varying behaviours are observed in a constant environment, it would be only due to the various sources of noise. B) In a competing hypothesis, non-constant output is generated intrinsically by an initiator of behavioural activity. Note that the sources of noise have been omitted in B merely because their contribution may be small, compared to that of the initiator. C) "Closed-loop model", that is with a closed reafferent feedback loop, where a state estimator, cross-correlating sensory input with recent motor commands via an efference copy (EC) allows efficient behavioural control of incoming sensory data. From Maye *et al*., PLoS One 2007.

Cheng *et al*. 2010, Wu *et al*. 2011). These feedback loops and their postulated comparison with the efference copies (Fig.1.4C) might involve processes of short-term information storage which might share mechanisms with short-term associative memory formation.

Efference copies: Some neurons have two collateral branches, such that one collateral branch triggers the action, and the other branch provides a "copy" of the motor command to the brain. The existence of these efference copies, first proved in fly by von Holst and Mittelstädt (1950), is useful for an organism in order to anticipate the sensory modifications associated with an action, for example visual and proprioceptive inputs accompanying active movement, and to dissociate them from external stimulations that come in addition to the expected, self-induced modifications, such as appearance of an object in the visual field, obstacles in the movements, or consequences of operant behaviour (review Webb 2004, Crapse and Sommer 2008). A constructivist point of view considers that the formation of such forward predictive structures and inverse goal-oriented control structures is the major source leading to a conscious self in humans (Butz 2008). For operant conditioning, such architecture is necessary to allow the detection of coincidence between behaviour (efference copy) and a change in the state of the animal (sensory input).

Sensory input: In operant conditioning the valence of the outcome strengthens or weakens the willingness to perform the respective behaviour. The way this valence is mediated in *Drosophila* is not yet known, but studies on invertebrates (review in Brembs 2003) provided evidence of some shared and non-shared mechanisms of operant and classical conditioning. In *Aplysia*, an appetitive reinforcement pathway relies on the same dopaminergic oesophageal neurons for operant (Brembs *et al*. 2002) and classical conditioning (Lechner *et al*. 2000a and b, Mozzachiodi *et al*. 2003). This is similar in the monkey where dopamine neurons are activated for any positive stimulation, either in absence of any learning context or during classical or operant learning (reviewed in Schultz 2002). This raises the question whether appetitive and aversive reinforcements for operant conditioning are transmitted by aminergic neurons in *Drosophila* as it is the case for classical conditioning (Schwaerzel *et al* 2003). What seems clear, however, is that operant conditioning of *Drosophila* in a number of paradigms is independent from the mushroom bodies (Wolf *et al*. 1998).

Temporal comparison: When sensory input immediately follows the efference copy, a causal link between action and outcome is inferred. The animal might use this coincidence to adjust its behaviour in an operant way. The mechanism responsible for coincidence detection is currently being unraveled. In *Aplysia*, a type II-Adenylyl Cyclase seems to be the molecular detector of this coincidence, activating a PKA cascade (Lorenzetti *et al.* 2008). Moreover, the same neuron B51, responsible for biting behaviour, can be operantly or classically "pseudo-trained". After classical pairing, the excitability of this neuron is generally decreased excepted for the CS-induced excitation whereas after an operant procedure it is increased (Lorenzetti *et al.* 2006).. In *Drosophila*, a dissociation between operant and classical learning has been found at the molecular level: PKC is specifically involved in operant conditioning, Adenylyl Cyclase specifically required for classical conditioning (Brembs and Pendl 2008, see also Box 1.2). It is however unknown where along the motor or premotor circuit the reinforcing signal acts.

For those considerations studying operant conditioning in *Drosophila* larvae, an even simpler organism than adult *Drosophila* yet offering the same genetic tractability, would be of real interest. In Chapter 4, we address the question of operant conditioning abilities in *Drosophila* larvae. We considered the locomotion normally expressed by the larvae when displacing themselves in a cue-less environment (see Chapter 5) and chose to punish turns towards one side, leaving forward crawling and turning towards the other side unpunished. We used vibration of the substrate as punishment. To our knowledge this is the first attempt of operant training in *Drosophila* larvae, for which it is unknown to which extent their own behaviour is self-controlled and whether the molecular and/or cellular machinery necessary for operant conditioning is ready.

Box 1.2. Operant vs classical conditioning in *Drosophila*

In adult *Drosophila*, the behaviour at the flight simulator allows separating classical from operant conditioning (respectively denominated as 'World-' and 'Self-learning' by Brembs 2003, 2009a, 2011):

- For World-learning, the arena is illuminated alternatedely with green or blue light, one colour being associated with heat shock.

- During test the flies avoid the punished colour.

- This learning relies on the AC/cAMP/PKA pathway.
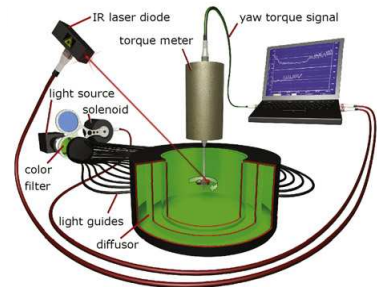
- For Self-learning, the arena is cue-less and yaw torque towards one side, either right or left, is associated with heat shock.

- As a result the fly avoids generating yaw torques towards the punished side during test.

- This learning is PKC dependent but independent of the AC/cAMP/PKA pathway; the mushroom bodies are dispensable for this form of conditioning.
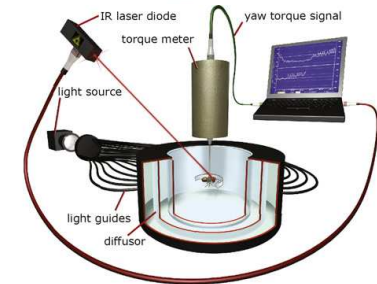
- Flies can also be trained in a composite way: yaw torque towards one side triggers illumination of the arena in one colour, and this is associated with heat shock, yaw torque towards the other side changes the colour of illumination and is safe.

- Tested for their colour preference, they avoid the previously punished colour. This learning is cAMP- and mushroom body-dependent.

- Tested for their yaw torque, they show no preference for any side. With a more intense training, or if mushroom bodies or cAMP function are defective, they lose their colour preference and gain a preference for a yaw torque side.

Self-learning test

WT	cAMP	MB	WT 16min
Impaired	OK	OK	OK

World-learning test

WT	cAMP	MB	WT 16min
OK	Impaired	Impaired	Impaired
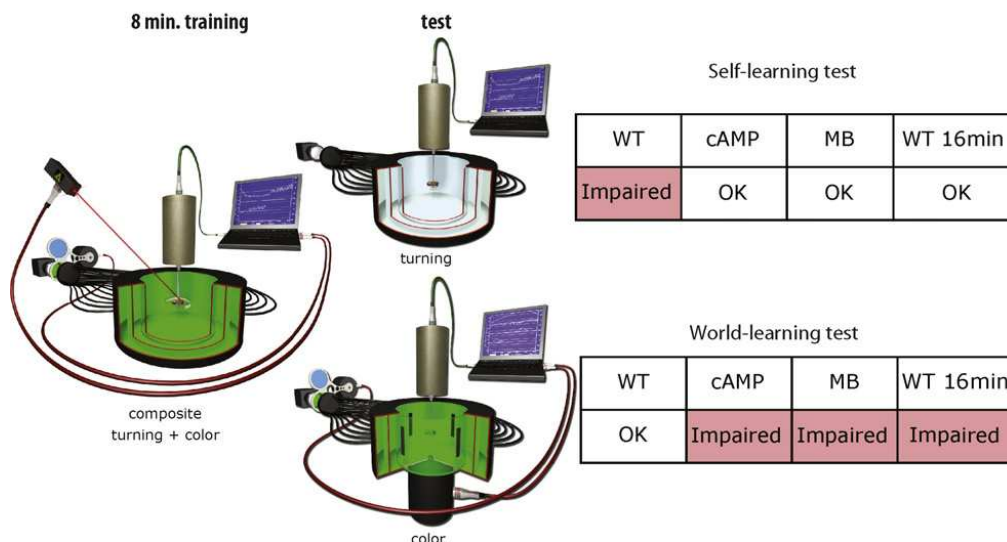
- According to the authors, the flies would learn with priority the rules governing events in the world ('World-' or classical learning), while learning about rules between the self and the world ('Self-' or operant learning), arguably mechanistically more demanding, would be inhibited by the mushroom bodies. This operant learning however would appear with repeated experience, *i.e.* intense training.

Chapter 2.[*]

Perception of Odour Mixtures in *Drosophila*

2.1 Introduction

"

The discovery of the Or family of olfactory receptors of *Drosophila* (Clyne *et al*. 1999; Vosshall *et al*. 1999; see also Benton *et al*. 2009 regarding the Ir receptor family) and ensuing neurogenetic analyses have led to a reasonably detailed picture of how different odours can cause different neuronal activity patterns along the olfactory pathway of insects (reviewed in Stocker 1994; Strausfeld and Hildebrand 1999; Galizia and Menzel 2000; Hallem *et al*. 2006; Vosshall and Stocker 2007; Gerber *et al*. 2009; Masse *et al*. 2009). Also, the short-term memory trace for olfactory associations with electric shock punishment has been localized to the mushroom bodies (reviewed in Heisenberg 2003; Gerber *et al*. 2004b; Krashes *et al*. 2007), a third-order ''cortical'' (Tomer *et al*. 2010) brain region of the insects, and the molecular nature of this trace is being characterized (reviewed in Davis 2004; Zars 2010). However, many questions remain, including how mixtures are processed, which is particularly relevant when considering that under natural conditions, animals always encounter volatile chemicals within mixtures or at least within substantial olfactory background.

On the physiological level, Silbering and Galizia (2007) compared patterns of calcium activity evoked by odours and their binary mixtures between the input and the output neurons (olfactory sensory neurons and projection neurons, respectively) of the *Drosophila* antennal lobe, the first relay of the olfactory pathway of the insects. The authors suggested both a global lateral inhibition acting as a gain control mechanism and specific inhibitory and likely also excitatory lateral connectivity, together leading to nonadditive processing of mixtures (a corresponding approach in honeybees also suggested that while on the level of olfactory sensory neurons there is little if any mixture interaction [Deisig *et al*. 2006], the projection neurons carry an olfactory representation that is not readily predictable by the activity patterns evoked by its components [Deisig *et al*. 2010]). Recent progress in the characterization of

local interneurons in the antennal lobe is now shedding light on exactly how these effects may come about (Chou *et al.* 2010; Huang *et al.* 2010; Yaksi and Wilson 2010). Although such analyses of the transfer functions within the microcircuit of olfactory sensory neurons, local interneurons, and the projection neurons certainly are indispensable to understand the physiology of mixture processing, it remained unclear how flies actually perceive mixtures relative to their component odours. Here, we take a behavioural approach toward this question.

We ask how strongly flies would avoid a mixture after punishment training with one of its constituent elements and how much, in turn, flies avoid an odour if it had been a component of a previously punished mixture. That is, we perform associative recognition experiments where a given single odour ''X'' is paired with an electric shock; then, conditioned avoidance of the flies toward a mixture containing X plus another odour ''1'' is measured. In independent sets of flies, the reverse is probed for, namely flies are trained with the mixture X1 and are tested with X. A distinguishing feature of our approach is that we adjust the dilutions of the used odours (benzaldehyde [B], 3-octanol [O], 4-methylcyclohexanol [M], and n-amylacetate [A]) for task-relevant behavioural potency, that is, for equal learnability (Niewalda 2010), rather than merely choosing odour dilutions that are physically the same or by adjusting for preference in experimentally naive animals (indeed, adjusting for equal behavioural effect of 2 odours in a given behavioural paradigm, suchas naive preference behaviour,does not necessarily entail equal behavioural effect in another paradigm such as learning [Saumweber *et al.* 2011a]). We specifically ask:

1. Is generalization between an odour and a binary mixture containing it symmetrical, that is, is conditioned avoidance equal if X is trained and the X1 mixture is tested, as when X1 is trained and X is tested?

2. Is an odour equally similar to different mixtures containing it, that is, is X equally similar to X1, X2, and X3?

3. Is a mixture equally similar to its constituent odour elements, that is, is X1 equally similar to X as it is to 1?

2.2 Material and Methods

2.2.1 Flies

Wild-type Canton-S flies were raised in groups of appr. 200, at 25°C, 60-70% relative humidity and a 14/10-h light/dark cycle. We collected flies one to five days after hatching from the pupal case, and kept them over-night at 18 °C until 24 h before the start of the experiment.

2.2.2 Stimuli and apparatus

We used four odours and their respective binary mixtures: benzaldehyde (B), 3-octanol (O), 4-methylcyclohexanol (M), and *n*-amylacetate (A) (CAS: 100-52-7, 589-98-0, 589-91-3, 628-63-7; all from Fluka, Steinheim, Germany, except A, from Merck, Darmstadt, Germany). Odours were diluted in paraffin oil (symbolized henceforth by Θ) (Merck) such that all odours supported statistically undistinguishable conditioned avoidance after odour-shock associative learning (Fig. 2.1 from Niewalda 2010) (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000); this equal learnability was confirmed within this study (Fig. 2.2B, B').
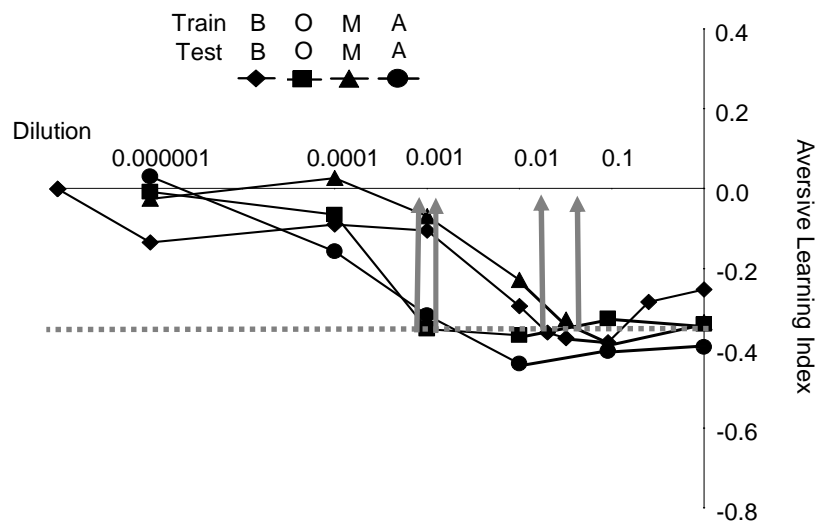
Figure 2.1. Adjustment of odour intensity for equal learnability. Flies are trained with a given odour at the indicated dilution, and then are tested using that same odour at that same dilution. Dilutions for further experiments are chosen such that learning indices are the same and, for each kind of odour, have just about reached asymptotic levels (stippled grey line and grey arrows) (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000). From the thesis of Niewalda, University of Würzburg 2010.

The training apparatus, modified from Tully and Quinn (1985), had been described in detail elsewhere (Schwaerzel *et al.* 2003, Yarali *et al.* 2008). In short, flies were transferred

into 90-mm long and 15-mm inner diameter plastic tubes, covered with an electrifiable copper grid to administer electric shocks during training (see below). These tubes were connected to Teflon containers for odour delivery by means of a suction pump that drew fresh air across the tube and ensured removal of odour-saturated air from the training apparatus. For single-odour presentation, 130 µl of odorant was applied in a 7-mm diameter Teflon cup. For the presentation of binary mixtures, twin cups were used which allowed separately loading two such volumes, such that the total surface for evaporation was doubled.

2.2.3 Principle of Training and Testing

Training was performed in dim red light, testing in darkness. In the following, we refer to Table 2.1 and use group 9a as an example: At t = 0 min, approximately 100 flies were loaded to the training tube. At t = 2 min, odour O was applied for 60 s. At t = 2 min 15 s, electric shock was applied for 60 s (90 volts, 12 pulses à 1.2 s, with an onset-onset interval of 5 s). At t = 4 min, Θ was presented for 60 s. Flies were left in the training tubes for recovery until at t = 9 min, when they were transferred back to their food vials for 13 min until the next of a total of three such training cycles started.

Once this O_{Shock}/Θ training was complete, the regular 13 min break was given until animals were loaded again to the apparatus for testing. After an accommodation period of 4 min, animals were transferred to the choice point of a T-maze, where they could escape towards either BO or Θ. After 2 min, the arms of the maze were closed and the number of animals within each arm (denoted # in the following) was counted. A preference score was calculated as:

$$\text{PREF } O_{Shock}/\Theta = (\#BO - \#\Theta) / \#Total \qquad (1a)$$

After one such score had been obtained, a second set of flies was trained reciprocally, such that electric shock was applied upon presenting Θ, but not upon presenting odour O (O/Θ_{Shock} training; Table 2.1, group 9b). Again, choice between BO and Θ was measured and a preference score determined:

$$\text{PREF } O/\Theta_{Shock} = (\#BO - \#\Theta) / \#Total \qquad (1b)$$

The preference scores are documented in Fig. 2.2A. From the preference scores of the two reciprocally trained sets of flies, a learning index (Fig. 2.2B, group 9) was calculated as:

$$LI = (\text{PREF } O_{Shock}/\Theta - \text{PREF } O/\Theta_{Shock}) / 2 \qquad (2)$$

Thus, positive LIs indicate conditioned approach, negative LIs conditioned avoidance. Note that across independent measurements, the sequence of events was either as indicated during

all three training cycles (*e.g.* first O-shock and then Θ), or was reversed (*i.e.* first Θ and then O-shock) (in the reciprocally trained sets of flies either first shock-Θ and then O, or first O and then shock-Θ). Flies were trained and tested only once. For all other groups listed in Table 2.1, experiments were performed and analysed accordingly.

Data are presented as box plots with the middle line showing the median and box boundaries and whiskers the 25%/ 75% and 10%/ 90% quantiles, respectively, and are analysed with non-parametric statistics (Statistica, Statsoft, Hamburg, Germany). We used non-parametric tests for statistical comparison: Kruskal-Wallis test (KW-test) and Mann-Whitney U test (MWU-test) were used for between-groups comparisons, and One-Sample-Sign test (OSS-test) was used for comparing scores to zero. In cases of multiple comparisons, we used a conservative approach by employing a Bonferroni correction to maintain the experiment-wide error rate at 5 %. That is, we divided P= 0.05 by the number of comparisons made, such that if *e.g.* three comparisons were made, P< 0.05/3 was used for each individual comparison. The respectively employed cut-off is indicated in the legends.

2.2.4 Experimental rational

To test how similar flies regard a binary mixture to one of its elements, we trained flies with an element X and tested them with a mixture containing it (X1), or trained them with a mixture X1 and tested them with one of its elements (either with X or with 1). The more similar the flies regarded the trained and tested olfactory stimulus, the higher the obtained score should be. From the 4 odours we used, we can thus draw 32 experimental groups (Table 2.1).

Given that in this approach we compared behaviour towards a mixture with behaviour towards an element contained in it, we first needed to see whether 2-fold differences in the total amount of odour between training and test would confer any asymmetry to this comparison. Therefore, in the case of the first 8 experimental groups listed in Table 2.1, we trained groups of flies with a single quantity of odour – *i.e.* using single odour cups as mentioned above –, and tested them with the double quantity– *i.e.* using twin odour cups – of that same odour (*e.g.* train B, test BB: Table 2.1, group 1); or we trained flies with a double quantity of odour, and tested them with a single quantity (*e.g.* train BB, test B: Table 2.1, group 2).

For the following 24 experimental groups (Table 2.1, groups 9 to 32), we either trained flies with an element, and tested them with a mixture containing it (*e.g.* train O, test BO: Table 2.1, group 9; or train B, test BO: Table 2.1, group 25); or we trained flies with the mixture, and tested them with one of its elements (*e.g.* train BO, test O: Table 2.1, group 10, or train BO, test B: Table 2.1, group 26).

Group	Punished*	Not punished*	Test	Group	Punished*	Not punished*	Test
1a	B	Θ	BB vs Θ	17a	A	Θ	BA vs Θ
1b	Θ	B	BB vs Θ	17b	Θ	A	BA vs Θ
2a	BB	Θ	B vs Θ	18a	BA	Θ	A vs Θ
2b	Θ	BB	B vs Θ	18b	Θ	BA	A vs Θ
3a	O	Θ	OO vs Θ	19a	O	Θ	OA vs Θ
3b	Θ	O	OO vs Θ	19b	Θ	O	OA vs Θ
4a	OO	Θ	O vs Θ	20a	OA	Θ	O vs Θ
4b	Θ	OO	O vs Θ	20b	Θ	OA	O vs Θ
5a	M	Θ	MM vs Θ	21a	B	Θ	BM vs Θ
5b	Θ	M	MM vs Θ	21b	Θ	B	BM vs Θ
6a	MM	Θ	M vs Θ	22a	BM	Θ	B vs Θ
6b	Θ	MM	M vs Θ	22b	Θ	BM	B vs Θ
7a	A	Θ	AA vs Θ	23a	M	Θ	BM vs Θ
7b	Θ	A	AA vs Θ	23b	Θ	M	BM vs Θ
8a	AA	Θ	A vs Θ	24a	BM	Θ	M vs Θ
8b	Θ	AA	A vs Θ	24b	Θ	BM	M vs Θ
9a	O	Θ	BO vs Θ	25a	B	Θ	BO vs Θ
9b	Θ	O	BO vs Θ	25b	Θ	B	BO vs Θ
10a	BO	Θ	O vs Θ	26a	BO	Θ	B vs Θ
10b	Θ	BO	O vs Θ	26b	Θ	BO	B vs Θ
11a	O	Θ	OM vs Θ	27a	M	Θ	MA vs Θ
11b	Θ	O	OM vs Θ	27b	Θ	M	MA vs Θ
12a	OM	Θ	O vs Θ	28a	MA	Θ	M vs Θ
12b	Θ	OM	O vs Θ	28b	Θ	MA	M vs Θ
13a	B	Θ	BA vs Θ	29a	A	Θ	OA vs Θ
13b	Θ	B	BA vs Θ	29b	Θ	A	OA vs Θ
14a	BA	Θ	B vs Θ	30a	OA	Θ	A vs Θ
14b	Θ	BA	B vs Θ	30b	Θ	OA	A vs Θ
15a	A	Θ	MA vs Θ	31a	M	Θ	OM vs Θ
15b	Θ	A	MA vs Θ	31b	Θ	M	OM vs Θ
16a	MA	Θ	A vs Θ	32a	OM	Θ	M vs Θ
16b	Θ	MA	A vs Θ	32b	Θ	OM	M vs Θ

Table 2.1. Summary of experimental groups. Description of all different training and test regimen. We used benzaldehyde, 3-octanol, 4-methylcyclohexanol, and *n*-amylacetate at single amounts (B, O, M, A), double amounts (BB, OO, MM, AA), or as binary mixture (BO, BM, BA, OM, OA, MA). In all cases, two reciprocal groups were trained, one receiving the shock in association with the odour, (*e.g.* odour B: group 1a) and presentation of the solvent (denoted as Θ) without shock, while the reciprocal group experienced the reverse contingency (odour B was applied alone, and the shock was delivered with the solvent: group 1b). A learning index is calculated as the difference in odour avoidance between these reciprocally trained groups. *Note that within all groups the sequence of trials was as indicated in half of the cases (*e.g.* first B-shock, then Θ), whereas in the other half of the cases it was reversed (*e.g.* first Θ, then B-shock).

2.2.5 Physico-chemical distance

We used the 184 physico-chemical properties that have been used previously (Schmuker and Schneider, 2007) using MOE, the Molecular Operating Environment (Chemical Computing Group, Montreal, Canada). Since the exact three-dimensional conformation of the odorant which is required to elicit receptor responses is not known, we

included only those properties that are independent of conformation (2D features). The features were scaled to a mean of zero and a variance of one (unit variance) with respect to the original data set used in Schmuker and Schneider (2007). Specifically, we calculated the mean of each feature, such as the number of bonds in the longest chain, over all 836 monomolecular compounds from the 2004 Sigma-Aldrich *Flavors and Fragrances* catalog, and subtracted this mean from the value of each individual compound. This was done separately for each of the 184 features, such that the average for each feature over the 836 compounds was zero. Similarly, we calculated the variance of each feature and divided the values of each individual compound by it, such that the variance of each feature was one (we used the same scaling factors also to scale the features of M, which had not been included in the original data set). Physico-chemical distances between odorants were then calculated using the L_1 distance measure ("manhattan distance": sum of absolute coordinate differences).

2.3 Results

2.3.1. Generalisation between element and mixture is partial and symmetrical

We asked for the perceptual difference between binary mixtures and their constituent elements. We tested flies' behaviour towards a mixture after having been trained with one of its elements, or their behaviour towards an element after having been trained with the mixture. Given that each element was presented at its elemental quantity in the mixture, such that the total amount of odorant was doubled in the mixture, we first tested whether the same learning scores were obtained after training with a single quantity of odour (*e.g.* B) and testing with the double quantity (*e.g.* BB), or if we used the double quantity for training and the single quantity for test (Fig. 2.2B). We found that scores were equal in all cases (Fig. 2.2B; MWU-tests, P> 0.05/4 for groups 1-8) (this is non-trivial, because over wide concentration ranges at least, odour intensity can be a major determinant for associative recognition [Yarali *et al.*, 2009]). We thus could pool the respective pairs of groups for further analysis. Using these pooled data, a comparison across all four odours did not reveal significant differences in learnability (Fig. 2.2B'; KW-test, H= 7.35, P> 0.05/2, df= 3), allowing us to estimate the baseline level of learning scores for the olfactory stimuli in this experimental series by the stippled grey line in Figures 2.2-2.4. We could thus ask whether, under such conditions of adjusted learnability, the similarity between element and mixture is symmetrical.
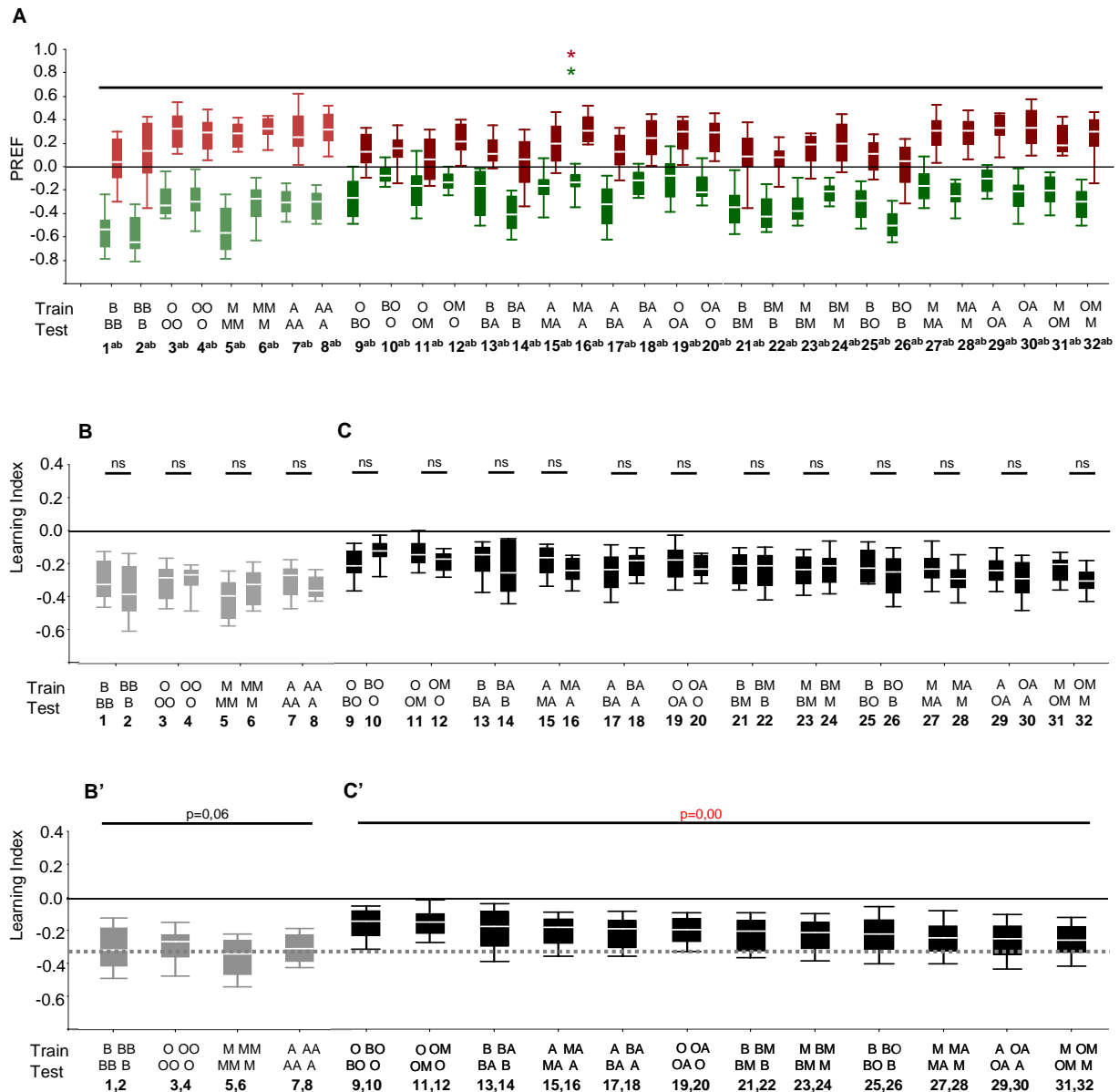
Figure 2.2. Symmetry of perceived distances. Preference scores (PREF, according to equations 1a,b) and learning indices dependent on the combination of odours for training and test. The respective training and test regimen and the group names according to Table 2.1 are indicated below the boxes. **A.** The preference scores differ between the a-type groups (green boxes: Kruskal-Wallis test, H [31, N= 731]= 267.8, P< 0.05) as well as between the b-type groups (red boxes: Kruskal-Wallis test, H [31, N= 731]= 161.4, P< 0.5); this holds true also when considering groups 1-8 separately from groups 9-32. **B.** Complementary groups of flies were either trained with an element and tested with a double quantity of this element, or vice versa; in all cases, the resulting learning scores were equal between these complementary groups (MWU-tests, P> 0.05/4). Sample sizes are from left to right: 24, 19, 22, 25, 22, 20, 21, 22. **C.** Complementary groups of flies either were trained with an element and tested with a binary mixture containing it, or were trained to the mixture and tested with one of its constituent elements; in all cases, scores were equal between these complementary groups (MWU-tests, P> 0.05/12), arguing for symmetry in perceived distance between element and mixture. Sample sizes are from left to right: 24, 20, 22, 23, 23, 22, 23, 21, 20, 20, 28, 23, 24, 22, 23, 20, 24, 24, 24, 21, 22, 26, 22, 21. In **B' and C'**, the pooled scores of the complementary groups from (**B**) and (**C**) are presented. Note that learnability in (**B'**) was statistically equal across the dataset (KW-test, P> 0.05/2), such that the stippled line, representing the median of the pooled data from (**B'**), could serve to indicate baseline learnability of the odours used. The significant difference of the scores in (**C'**) (KW-test, P< 0.05/2) argues that perceived distance between elements and mixture was different, depending on which odours are employed. All 12 odour pairs considered in (**C'**) showed scores different from zero (OSS-tests at P< 0.05/12), and had a score lower than the baseline (MWU-test, P< 0.05/12), except the score obtained from A and OA (MWU-test, P> 0.05/12); this argues for a usually partial generalisation between mixtures and their elements.

The same learning scores were found when training flies with *e.g.* B and testing them with BO, or when training them with BO and testing them with B (Fig. 2.2C; group 25 versus group 26); the same was found for all other element-mixture pairs (Fig. 2.2C; MWU-tests, $P> 0.05/12$ for groups 9-32). After pooling the respective element-mixture scores, it turned out that learned avoidance was observed to a significant extent (Fig. 2.2C'; OSS-tests against zero, $P<0.05/12$); this generalised learned avoidance, however, was partial, as in almost all cases (A and AO being the exception: Fig. 2.2C', groups 29, 30) scores were reduced as compared to baseline learning scores (Fig. 2.2C'; MWU-tests: $P<0.05/12$ in all cases excepted for A and AO). Thus, the flies regarded the mixture as similar to its elements- rather than as absolutely identical nor as totally different from it. Notably, the level of generalised learned avoidance varied across the considered element-mixture pairs (Fig. 2.2C; KW-test, $P< 0.05/2$, $H= 41.45$, $df= 11$).

2.3.2. An element is equally similar to all binary mixtures containing it

We compared the perceived distances between an element X and the three binary mixtures containing it (X1, X2, X3) (Fig. 2.3A, B). We did not see any significant difference regarding any of the four odours (Fig. 2.3A; KW-tests, $P> 0.05/4$ in all cases). In other words, adding any of the three odours to the 'centre odour' X results in perceptually displacing the mixture to about the same extent (denoted as radius *r* in Fig. 2.3B). Note, however, that the particular distance the mixtures have from X can be different depending on odour: The element O was perceived as more distant from all mixtures containing it than the other elements (Fig. 2.3C; KW-test $P< 0.05$, $H= 26.92$, $df= 3$; all pair-wise MWU-tests $P> 0.05/6$, except for the ones involving O, where $P< 0.05/6$). In other words, O had less impact on mixture perception than the other odours, an effect which was also seen in the following analysis.
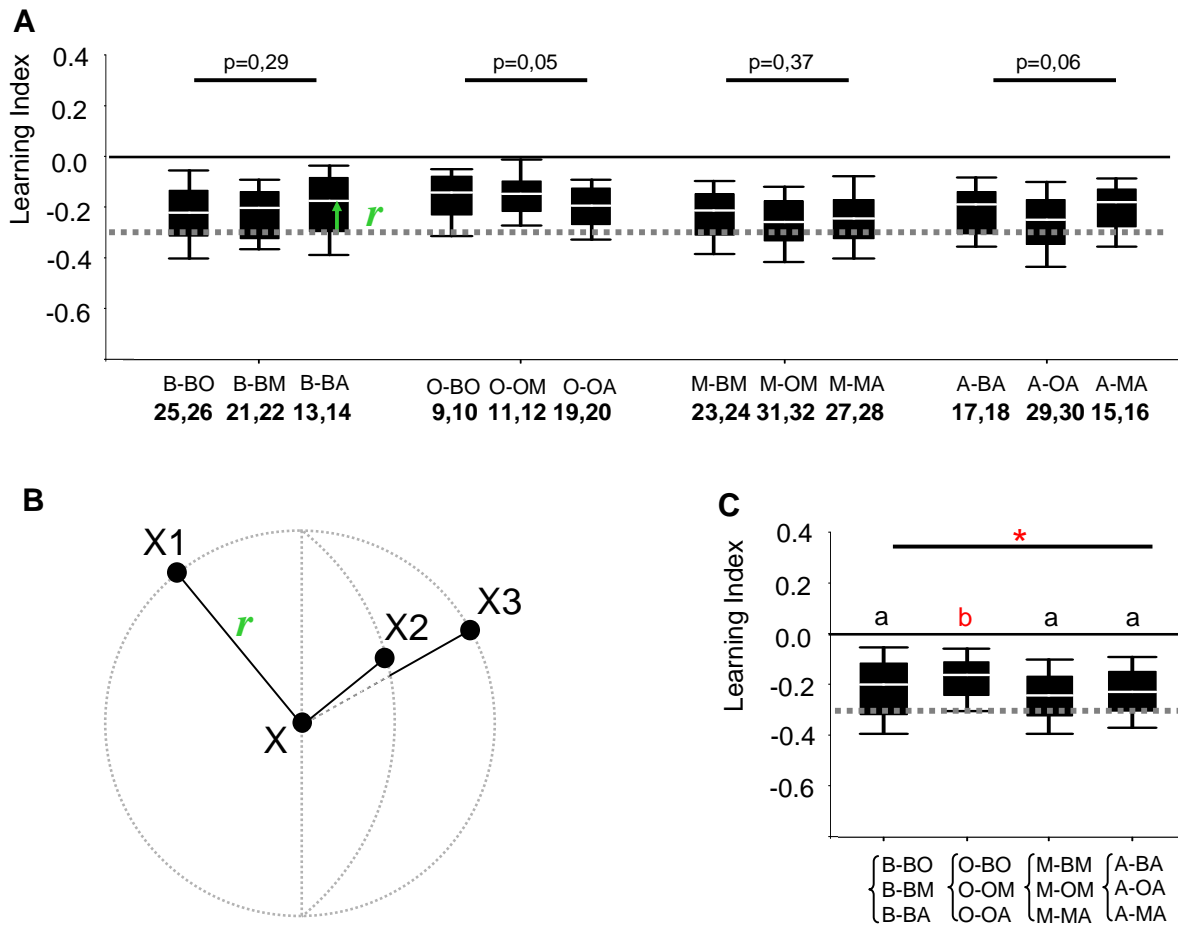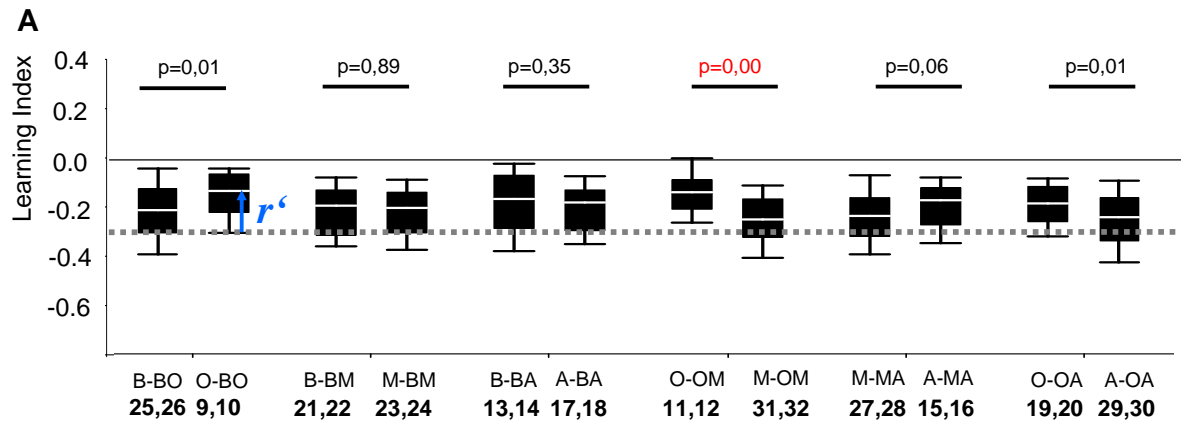
Figure 2.3. Perceived distances between an element and the mixtures containing it. A. Data from Fig. 2.2C' are organised to test for differences in perceived distance between a given element and the 3 possible binary mixtures containing it. In all cases, these distances were not significantly different (KW-tests, P> 0.05/4), arguing that the impact of a given element is similar regardless of its companion element in the mixture. This is represented by the sketch in (**B**). **C.** Data from (**A**) are pooled to allow testing whether the distance between elements and mixture differ between odours; it turned out that O is more distant to the mixtures containing it than any of the other elements (KW-test P< 0.05; shared or different lettering indicates MWU-tests with P> or < 0.05/6). For sample sizes, see legend of Fig. 2.2B, C; other details as in Fig. 2.2.

2.3.3. A binary mixture is equally similar to both constituent elements

Next, we asked for the distance between the mixture X1 and its constituent elements (*i.e.* X and 1) (Fig. 2.4). We found that in all cases, except for OM as a mixture, the elements were at about equal distance (denoted as *r'* in Fig. 2.4B) to the mixture (Fig. 2.4A; MWU-tests in all cases P> 0.05/ 6, except OM where P< 0.05/6). In other words, as a rule, both elements contribute about equally to mixture perception (Fig. 2.4B).
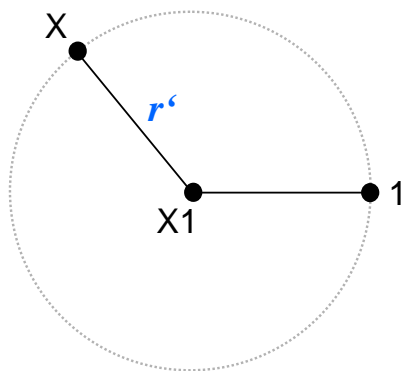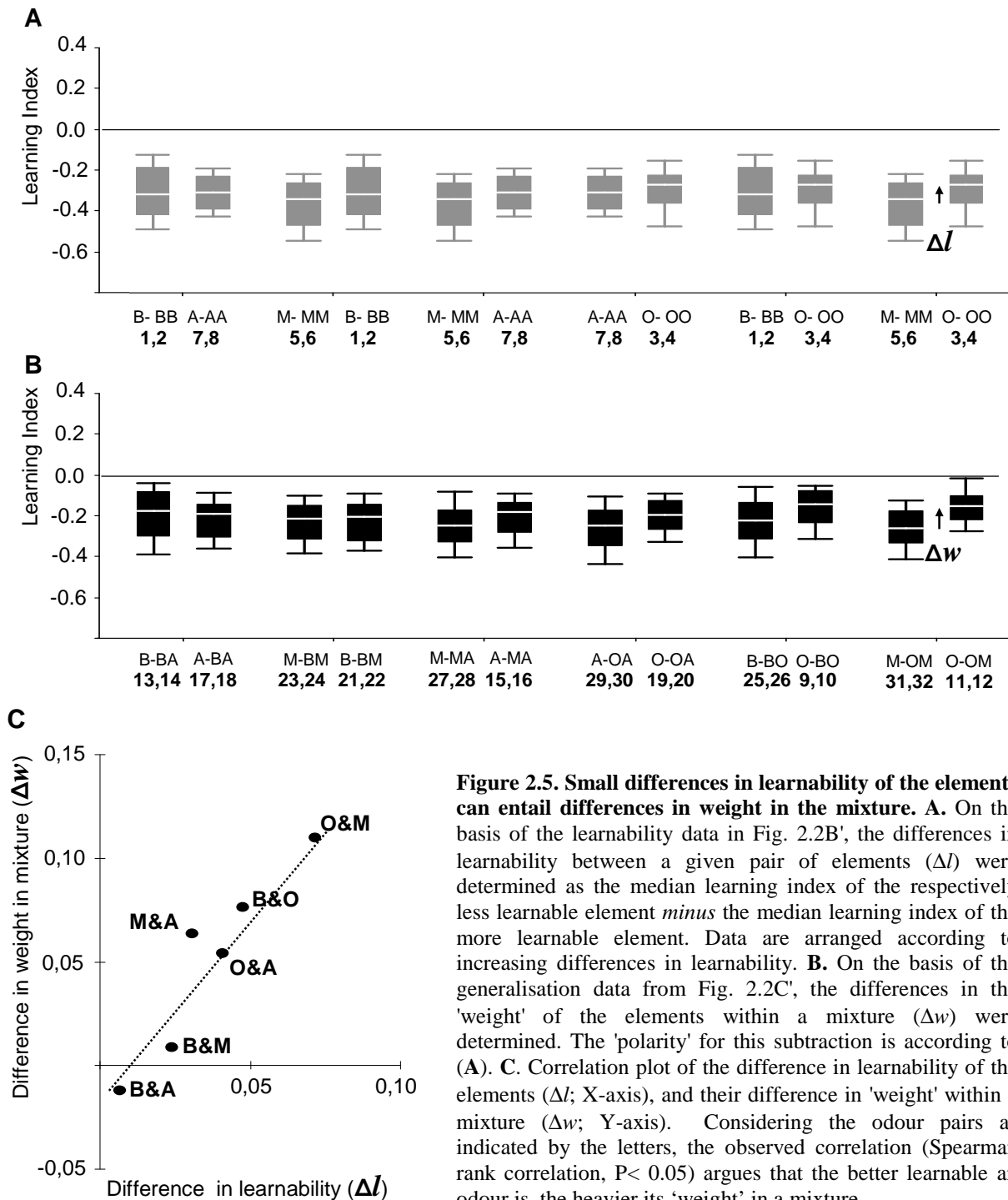
Figure 2.4. Perceived distances between a mixture and its constituent elements. A. Data from Fig. 2.2C' are rearranged to compare the distances between a mixture and either of its constituent elements. As a rule, the distances of either element to the mixture were equal (MWU-tests, P> 0.05/6); this rule is sketched in (**B**). The exceptional case was that the distance between O and OM was larger than the distance between M and OM (MWU-test, P< 0.05/6); in other words, the 'weight' of M in the mixture was higher than the weight of O in the mixture.

The exceptional case of OM, as well as the corresponding trends for the cases of BO and OA which just fall short of the Bonferroni-corrected statistical cut-off, prompted the question whether the learnability of an element accounts for its 'weight' in the mixture. Specifically, we asked whether if a given odour was more learnable than the other, that more learnable odour would also have the higher 'weight' in the mixture.

To this end, we first calculated the difference in learnability (Δl) between any pair of odours as the difference between the median learning index for the less learnable element minus the median learning index for the more learnable element; in the case of O and M, for example, the median learning index for M was more negative than for O (Fig. 2.5A, C). Second, we correspondingly calculated the difference in 'weight' in the mixture (Δw) (Fig. 2.5B); for the example of O and M, this revealed than the 'weight' of M in the OM mixture was higher than the 'weight' of O (Fig. 2.5B, C). After doing so for all cases, we found that these differences correlated (Fig. 2.5C; Spearman rank correlation: r= 0.94, t(N-2)= 5.66, P= 0.005). This suggests that even small increases in learnability of an odour can fairly strongly

increase its impact in a mixture containing it. The better learnability is being adjusted, however, the more do differences in 'weight' disappear.

Figure 2.5. Small differences in learnability of the elements can entail differences in weight in the mixture. A. On the basis of the learnability data in Fig. 2.2B', the differences in learnability between a given pair of elements (Δl) were determined as the median learning index of the respectively less learnable element *minus* the median learning index of the more learnable element. Data are arranged according to increasing differences in learnability. **B.** On the basis of the generalisation data from Fig. 2.2C', the differences in the 'weight' of the elements within a mixture (Δw) were determined. The 'polarity' for this subtraction is according to (**A**). **C.** Correlation plot of the difference in learnability of the elements (Δl; X-axis), and their difference in 'weight' within a mixture (Δw; Y-axis). Considering the odour pairs as indicated by the letters, the observed correlation (Spearman rank correlation, P< 0.05) argues that the better learnable an odour is, the heavier its 'weight' in a mixture.

2.4 Discussion

This study, based on associative recognition experiments, provides three relatively simple rules for the processing of binary mixtures in *Drosophila*. If the odour elements X, 1, 2, and 3 are adjusted for equal learnability, we found that (i) generalisation between a binary mixture and either of its elements is symmetrical (Fig. 2.2C) and partial (Fig. 2.2C'); that (ii) the element X is equally similar to the mixtures containing it (Fig. 2.3B); and that (iii) the mixture X1 is equally similar to both its constituent elements (Fig. 2.4B). These results do not provide evidence for mixture-unique effects in *Drosophila* olfactory perception.

We would like to stress, though, that the boundary condition for the applicability of these rules, namely that learnability indeed is adjusted, is important. That is, although it appears as if there is more generalisation between M and the OM mixture than between O and the OM mixture (Fig. 2.4A), this can be accounted for by the slightly lower learnability of O (Fig. 2.2B'). Indeed, although variations in learnability across all four odours formally remain below statistical cut-off using the warranted Bonferroni correction (Fig. 2.2B'), we do observe a correlation between differences in learnability and differences in 'weight' of an element in the mixture (Fig. 2.5C). Thus, 'imperfections' in learnability adjustment uncover that even small differences in learnability may be able feign asymmetries in mixture processing.

Interestingly, on the physiological level asymmetries in the weight of odours in a mixture can be accounted for by the signal intensities evoked by the odour elements in the projection neurons (for the honeybee: Deisig *et al.*, 2010, loc. cit. Fig. 4). Correspondingly, Lapid *et al.* (2008) found that human judgements of the pleasantness of an odour mixture follow a linear model taking into account the pleasentness judgements of its constituent elements- weighted by their respective perceived intensities.

We were further wondering whether the similarity between the mixture X1 and its elements X and 1 depends on the physico-chemical similarity between X and 1 (Fig. 2.6A). Consider as an extreme case that X and 1 were practically identical in terms of their physico-chemical properties; then the flies would regard the X1 mixture effectively as XX, leading to a small perceptual distance between X and what comes across to the flies as "XX". Taking advantage of the physico-chemical descriptions of odours according to Schmuker and Schneider (2007), we find that the more distant X and 1 are in terms of their physico-chemical properties, the more distant the flies regard X1 from its elements X and 1 (Fig. 2.6B;

Spearman rank correlation: r= 0.9, t[N-2]= 3.58, P= 0.04). This could account for the variations seen in the distances between element and the different mixtures containing it as seen in Figure 2.4A, which however remain below statistical cut-off when using the warranted Bonferroni correction.
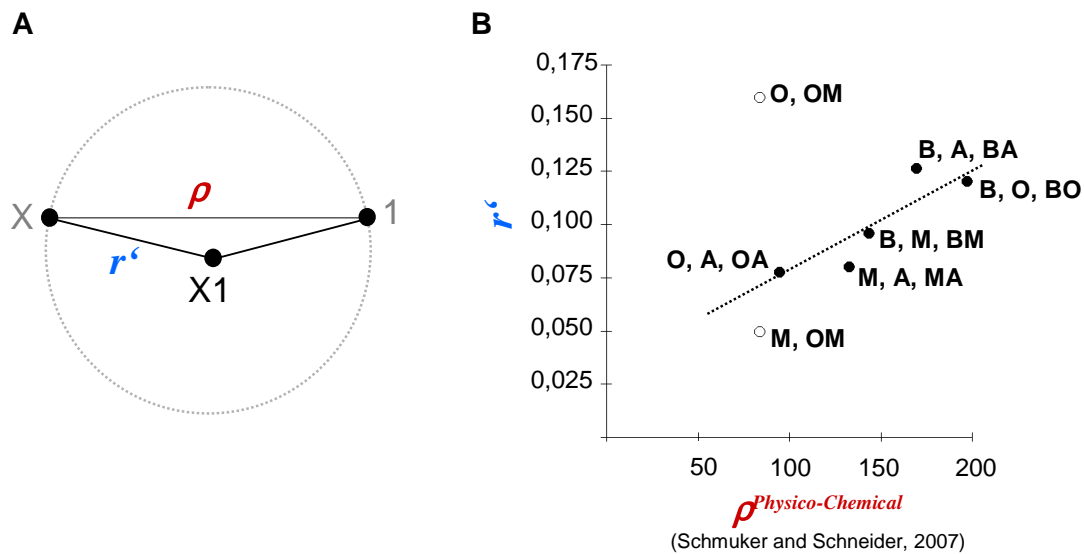
A

B

(Schmuker and Schneider, 2007)

Figure 2.6. Mixture-to-element distances correlate with element-to-element distances. A. Sketch to illustrate mixture-element distances (r') and element-element distances (ρ). **B.** The perceived distance between a mixture and its constituent elements (r'; Y-axis; data from Fig. 2.4A using the median of the pooled learning indices for each odour pair considered) can be predicted by the physico-chemical distance between the elements ($\rho^{Physico-Chemical}$: X-axis, data according to Schmuker and Schneider [2007]) (Spearman rank correlation: r= 0.9, P< 0.04). Data for O and M (open symbols) cannot be considered in the correlation analysis, as O and M are not equally learnable, leading to apparently asymmetric scores for r' when considering O-OM and M-OM (see Fig. 2.4A).

Contemplating once more that generalisation between element and mixture depends on the odour pairs considered (Fig. 2.2C), what are the determinants of generalisation? As argued above, both minor learnability differences and differences in physico-chemical relatedness are of influence, and may explain at least some variations in generalised conditioned avoidance. We further note that we do not find cases of no-generalisation, *i.e.* in no case is the mixture something totally different from the elements, nor do we typically see full generalisation. This latter observation is not trivial. Suppose recognition were determined by the mere presence of a learned element (for a discussion see Pearce, 1994), such that when testing with X1 the previously trained element X is recognized as such. Taken to its logical extreme, such a

scenario could predict full generalisation between elements and mixture, which is not observed (Fig. 2.2C'). This may imply that either after training with X this trained element X is recognized upon testing with X1, but its impact is scaled down because it is part of a mixture (as total odour amount does not have a systematic influence [Fig. 2.2B], scaling down according to total odour amount this does not seem likely). In turn, during training with X1, the elements may accordingly share into the training effects (again, Pearce [1994] includes a more detailed discussion). This process would be independent of the *kinds* of odour employed, but would require information about the *number* of monomolecular elements comprising the mixture being available to the olfactory system. Alternatively, with regard to the memory trace (which arguably is localized to the mushroom body Kenyon cells, a third-order 'cortical' brain region of the insects: Gerber *et al.* 2009), the neurons processing X may be overlapping with rather than being nested within the ones processing X1, such that depending on the *kinds* of odours employed only a fraction of neurons housing the memory trace can be activated. This would require lateral inhibition between the neurons activated by X and 1 at some point in the circuit upstream of the memory trace, potentially within the antennal lobe.

In conclusion, our results provide the first systematic account of mixture perception in *Drosophila*. We derive three rules of mixture perception: (i) mixture-element generalisation is symmetrical and partial; (ii) elements are equally similar to different mixtures containing it, and (iii) a mixture is equally similar to its elements. Importantly, we identify two boundary conditions for the applicability of these rules: First, the dilutions of the odours need to be adjusted for task-relevant behavioural potency, and second, the physico-chemical distances between the elements should be about equal. "

Chapter 3[**]

Classical Conditioning between Odours and Mechanosensory Punishment in larval *Drosophila*

3.2 Introduction
"

Drosophila melanogaster larvae can learn the association between odorants and gustatory reinforcement. Pairing either an odour (Scherer *et al*. 2003) or a light (Gerber *et al*. 2004) with appetitive substances such as fructose induces appetitive memory, while aversive memory is formed after pairing an odour with either a bad taste (quinine or highly concentrated salt, Gerber and Hendel 2006), or with electric shock (Aceves-Piña and Quinn 1979, Pauls *et al*. 2010a). In odour-reward learning for example, larvae are rewarded in presence of one odour, but not in presence of another odour (A+/B), and then are tested for their preference for A or B. A second group of larvae undergoes the same test, but after reciprocal training (A/B+). Thus, differences in test performance indicate an effect of the odour-reward contingency, in other words associative learning.

In terms of psychological mechanism, such conditioned behaviour reflects the expected outcome of tracking down the learnt odour: Conditioned search for reward in the appetitive case, and conditioned escape from punishment in the aversive case (Gerber and Hendel 2006; see also Schnaitmann *et al*. 2010). This interpretation is based on the observation that conditioned search is disabled if the sought-after reward is already present, and that conditioned escape is disabled if an escape-inducing punishment is not present (for a more detailed discussion see Schleyer *et al*. 2011).

In terms of neurobiological mechanism, odour-taste learning in the *Drosophila* larva has been analysed to some extent (Michels *et al*. 2005, Kaun *et al*. 2007, Zeng *et al*. 2007, Selcho *et al*. 2009, Pauls *et al*. 2010b, Michels *et al*. 2011, Saumweber *et al*. 2011b), based on the fairly detailed previous knowledge of the chemosensory pathways of *Drosophila* in

particular (reviewed in *e.g.* Scott 2005, Hallem *et al.* 2006, Gerber and Stocker 2007, Vosshall and Stocker 2007, Olsen and Wilson 2008, Gerber *et al.* 2009), as well as the progress in understanding olfactory learning in insects in general (reviews regarding *Drosophila e.g.* Heisenberg 2003, Keene and Waddell 2007, the honey bee Menzel 2001, Giurfa 2007, Schwärzel and Müller 2006, the cricket Mizunami *et al.* 2009). In brief, sensory neurons target the antennal lobes, a first-order brain region where lateral connections shape olfactory representations. Antennal lobe output neurons have two target areas. One collateral conveys olfactory information directly towards the lateral horn and further on towards premotor circuitry. The second branch involves a detour via the mushroom bodies and only then towards premotor circuitry. In contrast, gustatory information bypasses the actual central brain, and is conveyed from gustatory sensory neurons towards the sub-oesophageal ganglion and then to premotor centres in the ventral nerve cord. Notably, modulatory neurons ascending from the suboesophageal ganglion branch off towards the brain and in particular the mushroom bodies to signal internal reinforcement. Indeed, the mushroom bodies are the likely site of coincidence of olfactory and reinforcement information (Akalal *et al.* 2010, Gervasi *et al.* 2010). Notably, internal reinforcement is dissociated according to valence, such that the net training-effect of octopaminergic neurons, as defined by the TDC2-Gal4 expression pattern, is rewarding, and the net training-effect of dopaminergic neurons, as defined by the TH-Gal4 expression pattern, is punishing (Schroll *et al.* 2006; but also see Selcho *et al.* 2009).

Here, we extend the scope of larval olfactory learning models by using mechanosensory disturbance as a punishment. This seems timely as mechanosensation is rather well analysed (Jarman 2002, Kernan 2007, Lumpkin *et al.* 2010, Yin and Kuebler 2010, Wu *et al.* 2011), including attempts to unravel first- and second-order interneurons (Smith and Shepherd 1996, Diegelmann *et al.* 2008, Cardona *et al.* 2009). Also, from a practical point of view, temporal control over mechanosensory stimulation can be much finer-grained than is the case for gustatory reinforcement in the larva, where tastants have to be added to the substrate and therefore changes in substrate necessarily involve translocation of the animals.

Following the pattern of the models referred to above, one odour (A) is presented together with mechanosensory disturbance (a 'buzz': ◀), while another odour (B) is presented without such a disturbance (A◀/B training). Then, animals are offered the choice between A and B. A second experimental group is tested in the same way, however after reciprocal

training (A/B◀). We find that larvae show conditioned escape from the reinforced odour, indicating the punishing nature of the employed mechanosensory stimulus. We characterize basic parametric features of this model, including the movement kinematics with respect to the punishment, the temporal dynamics of retention during the test, the dependence of associative success on the number of punishment pulses within a trial, as well as on the number of training cycles, and on the amplitude of the mechanosensory disturbance. Last, but not least, we exploit this model to determine the rules of the behavioural expression of the memory trace.

3.3 Materials and Methods

3.3.1 Larvae, apparatus and stimuli

Larvae of the Canton-S wild-type strain (University of Würzburg) were raised in groups of appr. 200 at 25 °C, 60-70 % relative humidity, and a 14/10-h light/dark cycle. We used third-instar feeding-stage larvae throughout, aged 5 days after egg-laying.

Larvae in all experiments were free to crawl on a relatively large Petri dish (145 mm diameter; Starstedt, Nümbrecht, Germany) the bottom of which was covered with 1 % agarose (electrophoresis grade, Roth, Karlsruhe, Germany) on the eve of the experiment. This Petri dish was fixed on top of a loudspeaker (MC GEE 201847 CON Elektronik, Greußenheim, Germany, impedance 8 Ω, diameter 16 cm, acoustic pressure: 89.2 dB/W, power 150 W RMS) in a 50 x 50 x 75 cm box covered on its inside by silencing foam (Fig. 3.1A). The loudspeaker could be activated via a computer and was set to produce a vibration with a speed of displacement of 1.1 m/s, at a frequency of 100 Hz, unless mentioned otherwise. For punishment, 200-ms pulses of such vibrations were delivered once per second, unless mentioned otherwise (this stimulus is defined as 'buzz': ◀). A webcam (5 frames s[-1]) mounted above the Petri dish allowed recording of the larvae for offline analyses; to facilitate image acquisition, a ring of 30 red-light emitting diodes (624 nm LED; Conrad Electronics, Berlin, Germany) was arranged around the Petri dish. To ensure even dispersion of light, a 1cm-thick ring of opaque Perspex was inserted between these LEDs and the Petri dish. The over-all design of this set-up corresponds to the one reported by Wu *et al.* (2011).

As olfactory stimuli we used 1-octanol (OCT, purity 99%) and *n*-amyl acetate (AM, purity 98 %, diluted 1:50 in paraffin oil) (both Merck). We applied 10 μl of odour substance

onto each of two 7 mm² filter papers that were pasted inside the lid of the Petri dish, 5 cm from its edge and appr. 5 cm apart from each other along the equator of the dish. For better aeration, we used custom-made Petri dish lids perforated in the middle by 10 holes with 0.5mm diameter each.

For gustatory punishment, we used either 4 M of sodium chloride (NaCl, purity 99.5 %, Roth, Karlsruhe, Germany) or 0.20 % of quinine hemisulfate (QUI, purity 92 %, Sigma-Aldrich, Munich, Germany) in agarose for preparing the Petri dishes.

3.3.2 Learning protocol

We compared cohorts of 50 larvae that received reciprocal associative conditioning (Fig. 3.1B): For the first group, AM was presented together with the buzz, whereas OCT was presented alone (AM◀/OCT); for the second group, OCT was presented with the buzz and AM was presented alone (OCT◀/AM). After such training, larvae were tested for their preference for the two odours. A difference in AM-OCT preference between the reciprocally trained groups thus indicates associative learning.

Specifically, ~50 larvae were taken from their rearing vials, gently washed in tap water, and placed on a 145-mm diameter plastic Petri dish. Immediately before the beginning of each trial, odour (*e.g.* AM) was loaded and the lid of the training Petri dish was closed. Throughout the subsequent 5 min training trial, the buzz was applied (AM◀). Then, larvae were gently removed with a wet brush and placed on a fresh training Petri dish, this time loaded with OCT; during this trial, no buzz was presented (OCT). This AM◀/OCT training cycle was repeated three times. Between trials, the training Petri dish was discarded, while the odour-loaded filter papers were removed from the perforated lid which was then cleaned with alcohol, and equipped with freshly loaded filter papers for the following trial with that odour.

For testing, the larvae were transferred to the middle of a test-Petri dish containing agarose as usual, but offering a choice between AM on one side and OCT on the other side; unless mentioned otherwise, testing was carried out in the presence of the training-reinforcer, as this is required to reveal conditioned escape (Gerber and Hendel 2006, see also Schnaitmann *et al.* 2010). Larvae were allowed to wander in the test Petri dish for 5 min. At the time points mentioned in the Results, we counted the number of larvae on either side of

the Petri dish, and on a 1 cm-wide middle stripe (@AM, @OCT, @Middle). We calculate a preference index as:

$$(1) \quad PREF = (@AM - @OCT) / (@AM + @OCT + @Middle)$$

This preference index thus varies between 1 (indicating preference for AM), and -1 (indicating preference for OCT), while a preference index of 0 would indicate that the larvae distributed equally between the odours.

After one such preference value was obtained, a second cohort of 50 larvae was trained reciprocally (*i.e.* OCT◄/AM), and the choice behaviour was described by the preference score as detailed above. This allowed calculating an associative performance index (PI), quantifying the difference in preference between the reciprocally trained larvae:

$$(2) \quad PI = (Pref_{AM_{◄}/OCT} - Pref_{OCT_{◄}/AM}) / 2$$

PI thus also varies between 1, indicating conditioned approach, and -1, indicating conditioned avoidance.

Please note that in half of the cases the sequence of training trials was as indicated (*i.e.* AM◄/OCT and OCT◄/AM for the reciprocal groups), but in the other half of the cases the sequence of trials was reverse (*i.e.* OCT/AM◄ and AM/ OCT◄, respectively). The sequence of training trials had no significant influence on test behaviour (Fig. 3.3C).

For odour-taste learning, experiments were performed in the very same way as detailed above, except that either NaCl (4M, purity 99.5 %, Roth, Karlsruhe, Germany) or QUI (0.20 %, purity 92 %, Sigma-Aldrich, Munich, Germany) was used instead of the buzz.

3.3.3 Kinematics of larval movement

We used custom-designed tracking software in LabVIEW (National Instruments, Austin, Texas) to detect larvae by luminosity contrast. For each frame (frame rate $5s^{-1}$), we determined the position of the centroid of the larva and the orientation of the longitudinal axis going through it (Fig. 3.2A). From this information, we characterized the kinematics of the behaviour of the larvae upon presentation of a buzz:

- We calculated the speed (mm/s) of the larvae by considering their centroid during each of the respective one-second periods as the frame-to-frame sum of the distances covered by the centroid during that second.

- We calculated the angular speed of the larvae (°/s) as the frame-to-frame sum of the orientation changes of the longitudinal axis during the considered second.

For display purposes (Fig.s 3.2 and 3.5), we consider the relative speed and turning propensity, using the median value of the two seconds preceding the buzz of the considered individual as baseline. The absolute baseline values of median speed and median turning propensity are mentioned in the legends of the figures .

3.3.4 Statistics

Given the definition of the preference and PI scores, and given the fact that often these scores are not normally distributed, we opted for non-parametric statistics and display throughout. We used Kruskal-Wallis tests (KW-tests) for comparisons across multiple groups, followed in case of significance by pair-wise comparisons with Mann-Whitney U-tests (MWU-tests). One-sample sign tests (OSS-tests) were used to compare scores to zero. When multiple comparisons were made within one experiment, we applied a Bonferroni correction; that is, the criterion of significance (0.05) was adjusted by dividing it by the number of comparisons performed, such that the experiment-wide error remains below 5 %. All statistical tests were performed with Statistica 7.1 on a PC. Data are presented as box-whisker plots, with the middle bold line indicating the median, the box boundaries indicating the lower and the upper quartile, and the whiskers the 10 % and the 90 % percentile.
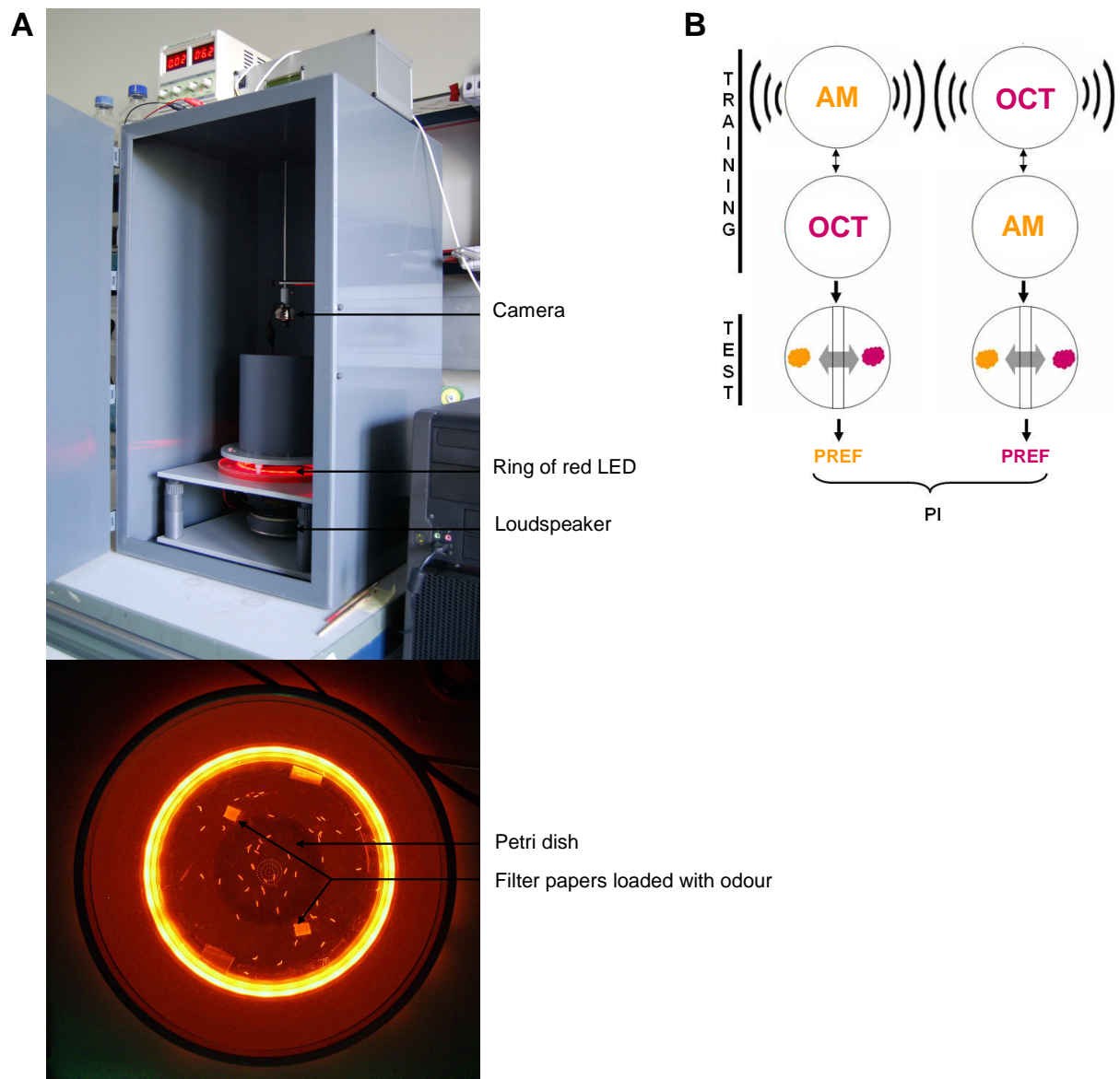
Figure 3.1. Paradigm for odour-buzz associative learning. A) Experimental arena: Inside a dark box illuminated with red LEDs, the larvae were free to crawl on a Petri dish with an odour emanating from odorant-soaked filter papers taped on the Petri dish lid. The Petri dish was fixed on top of a loudspeaker to deliver mechanosensory disturbances ('buzzes'). Larval behaviour was recorded by a webcam for offline analyses. **B)** Experimental design: During training, a first group of larvae received the buzz during the presentation of *n*-amylacetate (AM) while 1-octanol (OCT) was presented alone (AM◀/OCT). A second group received the reverse contingency (OCT◀/AM). These training cycles were repeated three times, unless specified otherwise. For the test, larvae were free to crawl on a test Petri dish for five minutes, with AM and OCT presented on opposite sides to create a choice situation. For both reciprocally trained groups, the preference for AM (Pref) was calculated. The associative performance index (PI) quantifies the difference in preference between the reciprocally trained groups, and thus associative learning (for details see Materials and Methods section), such that negative PIs indicate aversive memory, positive PIs appetitive memory. Please note that throughout this study the sequence of trials was as indicated in half of the cases (*i.e.* AM◀/OCT and OCT◀/AM), whereas it was reverse in the other half (*i.e.* OCT/AM◀ and AM/OCT◀, not shown).

3.4 Results

3.4.1 Behaviour of experimentally naïve larvae towards the buzz

We first describe the unconditioned behaviour of the larvae upon presentation of the buzz. Larvae were placed onto a Petri dish, and after one minute a single, 200 ms-buzz was presented. As parameters for analysis we chose the speed of the centroid of the larva (mm/s), and the larvas´ turning propensity (°/s) (Fig. 3.2A). As shown in Figure 3.2B, the buzz induced the larvae to slow down within the ensuing second (OSS-tests, $P > 0.05/5$ during the buzz and $P < 0.05/5$ for the four 1-s periods after the buzz, N= 122); with additional delay, larvae then increase turning propensity (Fig. 3.2C; OSS-tests, $P > 0.05/5$ during the buzz and during the first, second and fourth 1-s period after the buzz; $P < 0.05/5$ for the third 1-s period after the buzz).
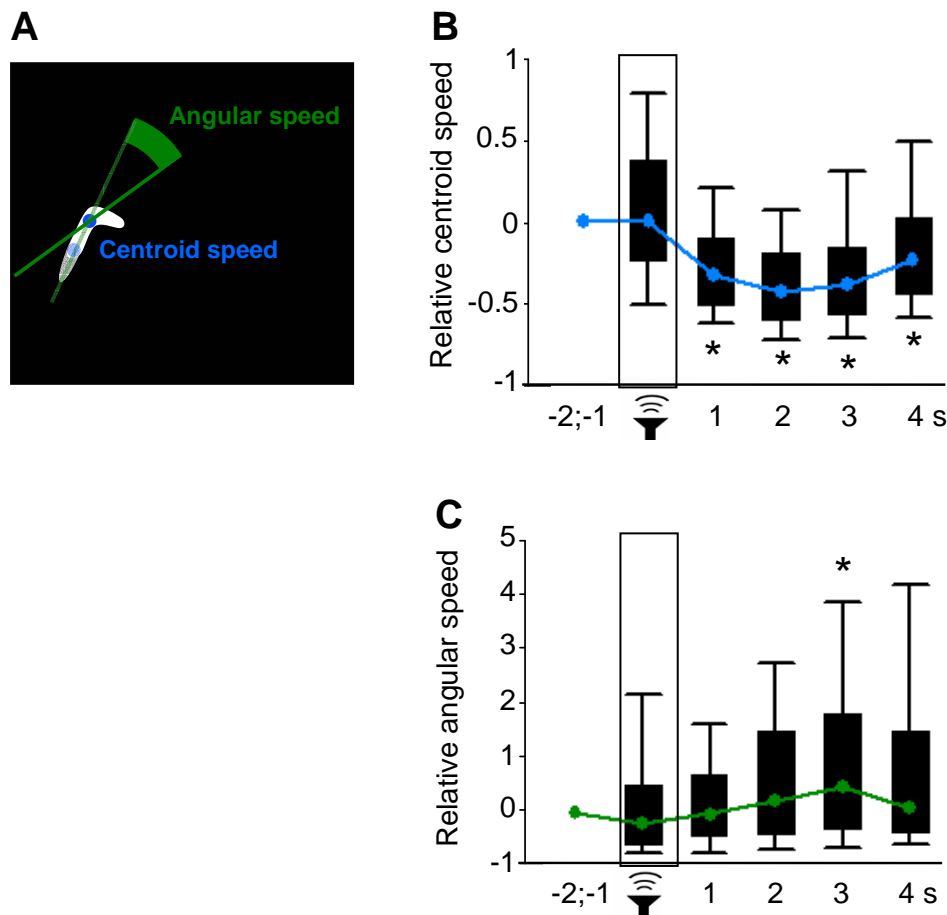
Figure 3.2. Unconditioned behaviour towards the buzz. (A) Sketch of the kinematic measures taken. We determined the speed of the centroid (B) and the turning propensity (C) of individual, experimentally naïve larvae for the 4 s following a buzz, relative to baseline [the median of respectively speed (0.76 mm/s) and angular speed (15.3 °/s) for the 2 s preceding the buzz]. Apparently, larvae slow down and then turn in response to a buzz. Asterisks stand for a significant difference of the scores [one-sample signe (OSS) test: $P < 0.05/5$] from baseline.

These results replicate the ones reported by Wu *et al*. (2011) using a similar experimental set-up. We interpret such buzz-induced behaviour (which is similar to what has been described in response to light 'touch': Kernan *et al*. 1994) as a startle response followed by reorientation, together comprising a behavioural 'escape' module. We therefore reasoned that the buzz may be effective as a punishment.

3.4.2 Establishing odour-buzz memories, and translating them into conditioned behaviour – or not

Larvae were trained either as AM◀/OCT or reciprocally as OCT◀/AM, and then were tested for their preference for AM and OCT (Fig. 3.3A). In Figure 3.3B, we display the resulting preference indices of these reciprocally trained larvae, for each minute of the 5 min-test. When tested in the absence of the buzz, odour preferences were equal between the reciprocally trained groups (Fig. 3.3B left; MWU-tests, $P>0.05/5$, U= 248, 242, 271.5, 260, 287 for the five testing periods, N= 24). In contrast, larvae tested in the presence of the buzz revealed associative memories between odours and buzz: We observed significant escape from the previously punished odour by the end of the second minute (Fig. 3.3B right; MWU-tests, $P> 0.05/5$, U= 212 for the first, and $P< 0.05/5$, U= 103.5, 63.5, 78, 62.5, for the second to the fifth testing minute; N= 24).

Considering Fig. 3.3B as well as the previous literature on odour-tastant learning, we decided to use the data from the end of the third testing minute for a calculation of the associative performance indices. It turns out that associative performance indices of larvae tested in the absence of the buzz were not different from chance (Fig. 3.3D; OSS-test, $P< 0.05/2$), but when tested in the presence of the buzz, we observed significantly negative associative performance indices (Fig. 3.3D; OSS-test, $P< 0.05/2$) (a direct comparison between the performance indices with a MWU-test yields $P< 0.05$, U= 146).

Given that the larvae tested in presence versus absence of the buzz have undergone the same training and thus must have stored the same odour-buzz memories, these results not only argue that odour-buzz associative memories are formed, but they also mean that, dependent on the testing situation, these memories can be 'translated' into conditioned behaviour – or not. Specifically, and as was previously reported for odour-bitter and odour-high-salt associations (Gerber and Hendel, 2006), aversive memories are behaviourally expressed in the

presence of punishment but not in its absence, and in this sense are embedded into an conditioned 'escape routine' which is employed only when escape indeed is warranted.
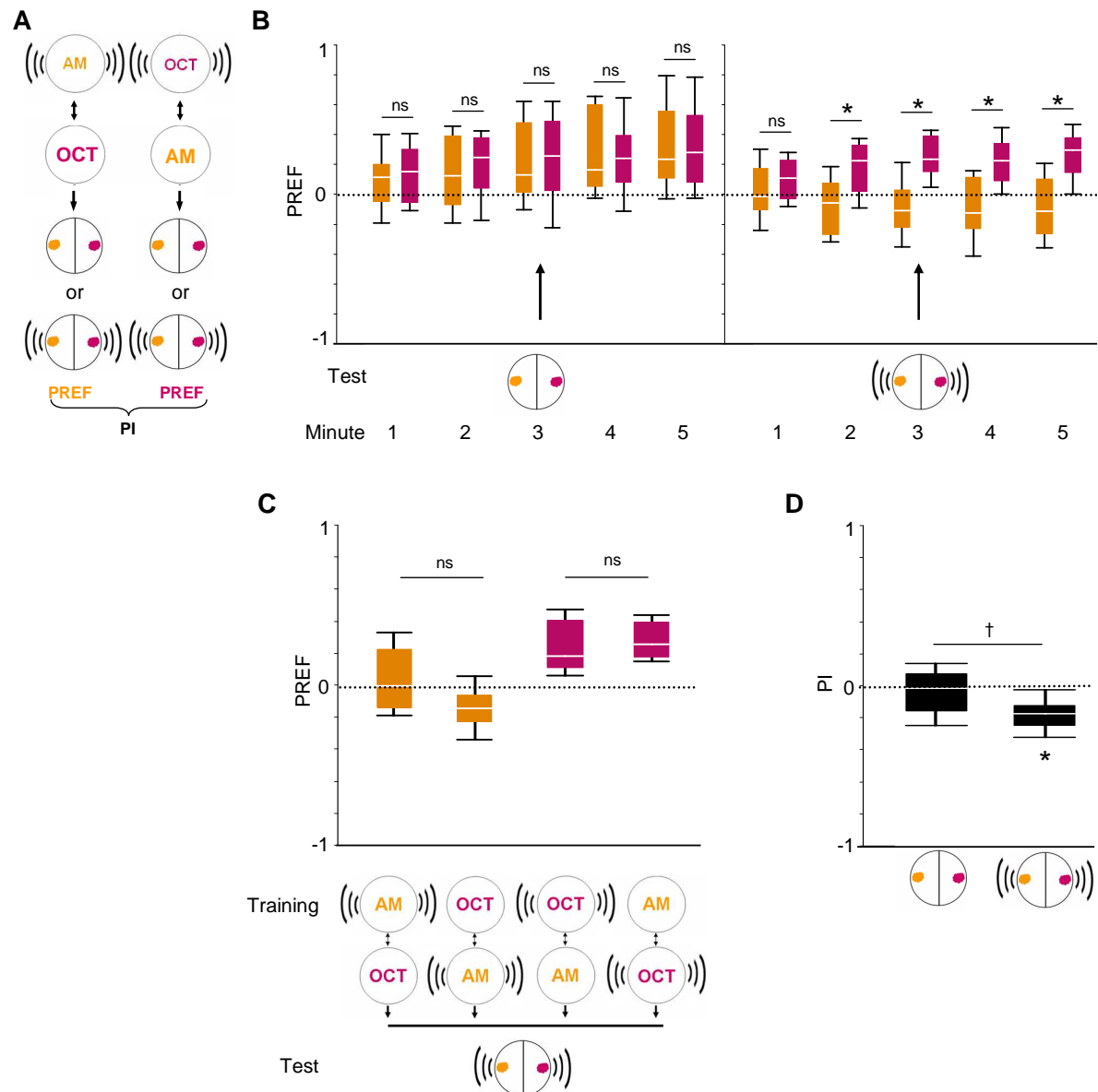

Figure 3.3. Odour-buzz associative learning. (A) Sketch of the experimental paradigm. Two groups of larvae underwent reciprocal odour-buzz training, and the difference in preference between them was quantified by the associative performance index (PI). Testing was carried out either in absence or presence of the training buzz. (B) For both reciprocally trained groups, the preference for AM is displayed separately for each minute of the 5 min test period. A difference in AM preference between groups needed at least 2 min to appear, and was observed only when the test was carried out in the presence of the training-buzz. Statistically significant differences between preference scores are indicated by asterisks (MWU-test: $P < 0.05/5$). C) These preferences scores did not vary according to the sequence of trials during training (*e.g.* whether training followed the sequence AM◀/OCT or OCT/AM◀. D) Associative performance indices obtained from the preference scores in (B), using the data from the third minute of test (arrow). Only when testing was carried out in the presence of the training-buzz, aversive memories were uncovered, as indicated by negative performance indices (OSS-test: $P < 0.05/2$).

3.4.3 More buzzes per trial – better learning

To parametrically characterize odour-buzz associative learning, we varied the number of punishment pulses by changing the interval between the buzzes from 0.4 s (corresponding to a total of 750 pulses per trial) to 125 s (2 pulses / trial, Fig. 3.4A). Independent groups of larvae were tested either in absence or in the presence of the respective training-buzz.

Confirming the previous results, associative performance indices were zero when the larvae were tested in the absence of the buzz (Fig. 3.4B; left-most plot; a KW-test across all groups tested in the absence of the buzz yields P> 0.05/2, H= 12.76, df= 6, N= 22, 25, 25, 29, 25, 25, 25; for the pooled data, the OSS-test yields P> 0.05/8, N= 176: Fig. S3.1). In contrast, aversive memories were revealed when testing in the presence of the buzz, and more importantly in the current context, the associative performance indices observed depended on the number of punishment pulses (Fig. 3.4B; for the groups tested in the presence of the buzz, KW-test: P< 0.05/2, H= 15.82, df= 6, N= 22, 25, 25, 25, 25, 25, 25). Specifically, performance indices remained below statistical cut-off as long as fewer than 60 pulses per trial were used (Fig. 3.4B; OSS tests: P> 0.05/8 in all three cases), but aversive memories were revealed for 60 or more pulses per trial (Fig. 3.4B; OSS-tests: P< 0.05/8 in all 4 cases) (for the underlying preference scores of this experiment: Fig. S3.2).
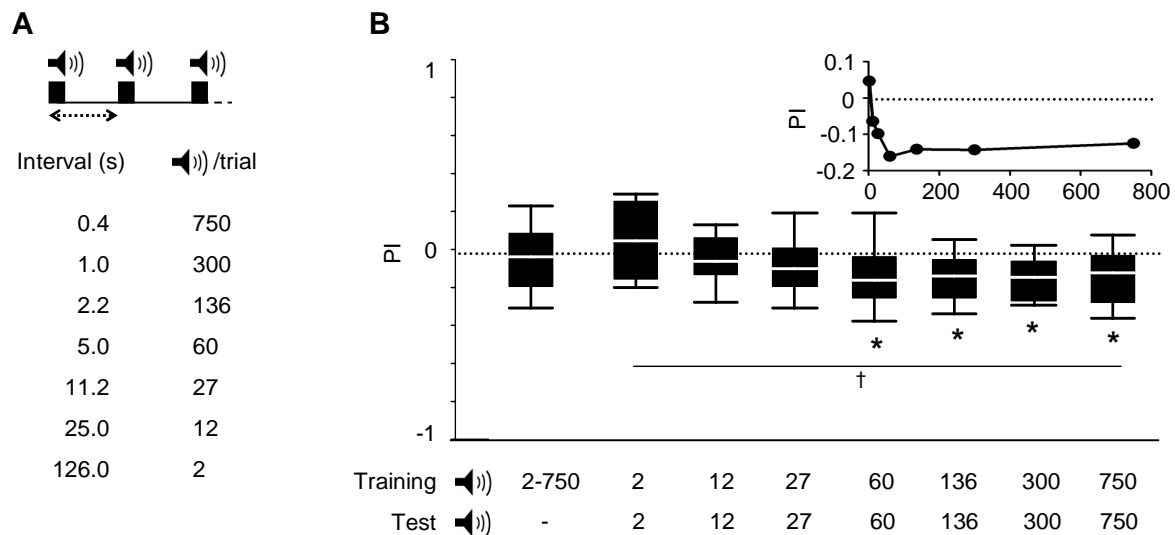

Figure 3.4. Increasing the number of punishment pulses per trial increases the associative effect. (A) Table of the parameters of punishment. (B) Irrespective of the number of punishment pulses per trial, odour-buzz memories are not behaviourally expressed when tested in the absence of the training buzz (leftmost plot). The data are thus pooled between groups (for non-pooled data, see Fig. S3.1). Testing in the presence of the training buzz, however, uncovers odour-buzz memories; notably, the associative effect increases with the number of punishment pulses per trial. The difference across groups is indicated by the lower dagger (KW-test: P<0.05/2). The inset displays the median associative performances indices plotted linearly across the number of buzzes per trial. The four right most groups have PIs significantly different from zero (asterisks, OSS-test: P< 0.05/8). For the underlying preference scores, see Fig.S3.2.

3.4.4 Interplay: Behaviour towards the buzz during test

At this point, we wondered whether the behaviour of the larvae towards the buzz would be associatively altered by the training regimen and/or would be changing across the 3 min testing period. We focused on two time-points: The very first buzz delivered during test (Fig. 3.5, left, N= 452), and the very last buzz delivered during test (Fig. 3.5, right, N= 432). For either time point, we separated the data according to whether the observed larva was located on the side of the previously punished odour or the previously non-punished odour.
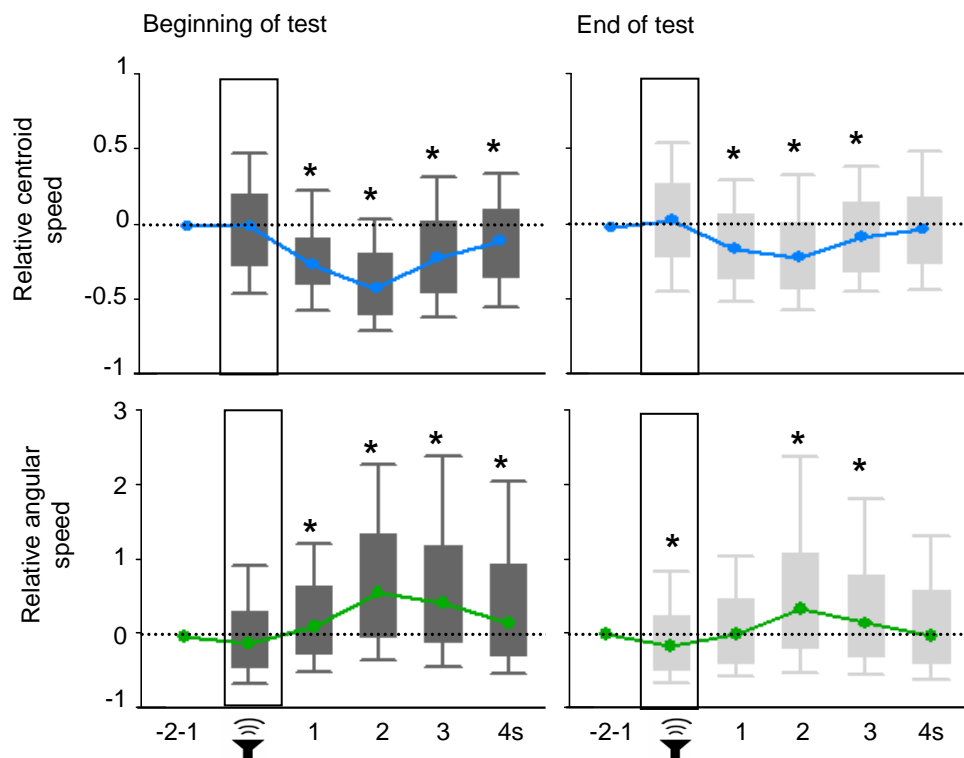
Figure 3.5. Behaviour towards the buzz during the test. Larvae were trained with 60 buzzes per trial and were then individually tracked during the test, which was carried out in the presence of the buzz. For the very first as well as the very last test-buzz (left and right column, respectively), we determined centroid speed (top row; mm/s) and turning propensity (bottom row; °/s). Scores are displayed from 2 s before to 4 s after the buzz. The data were normalized and statistically compared to the scores obtained for the respective individual during the 2 s preceding the buzz (median-values for speed: 1.13 mm/s at the beginning and 0.95 mm/s at the end of the test and turning propensity: 13.7 °/s at the beginning and 14.3 °/s at the end of the test) (asterisks for P< 0.05/5 in OSS-test).

It turned out that locomotor kinematics appear uniform regardless of experimental history of the ambient odour (not shown). Further, although the buzz induced a decrease in speed and an increase in turning both at the beginning and at the end of the testing period (Fig. 3.5; OSS-tests with P< 0.05/5 as criterion), speed decreased less and turning increased less at the end of testing (Fig. 3.5; all MWU-tests: P<0.05/5 for beginning versus end). Also,

we noted that the effect of the buzz on locomotion appears slightly diminished from what we had observed before for experimentally naïve larvae (compare Fig. 3.2 to Fig. 3.5, left). This suggests that buzz-induced escape behaviour, in terms of the slowing-down-and-turn behavioural components, although sensitive to non-associative changes, is in principle robustly observed even after up to 3x 60 presentations during 5 minutes of training, after odour exposure as entailed by the training regimen as well as experimental handling, plus the 48 buzzes received during testing. This, we believe, underscores its predominantly unconditional, reflexive character.

3.4.5 More training cycles – better learning

Returning to the parametric analyses of odour-buzz associations, we next asked whether associative performance indices would increase with extended training. To this end, we trained larvae with either 1, 2 or 4 training cycles (Fig. 3.6A). Using relatively mild punishment (60 buzzes per trial) revealed an increase in associative effect (Fig. 3.6B; KW-test: $P < 0.05/2$, $H = 8.34$, $df = 2$, $N = 16, 16, 16$) such that at least 2 training cycles were needed to reach significance (Fig. 3.6B; OSS-tests: $P < 0.05/3$ after 2 and after 4 training cycles). Interestingly, this incremental effect of the number of training cycles is obscured if more severe punishment is used (300 pulses per trial) (Fig. 3.6C; KW-test: $P > 0.05/2$, $H = 0.98$, $df = 2$, $N = 8, 8, 8$; the OSS-test for the pooled data yields $P < 0.05$).
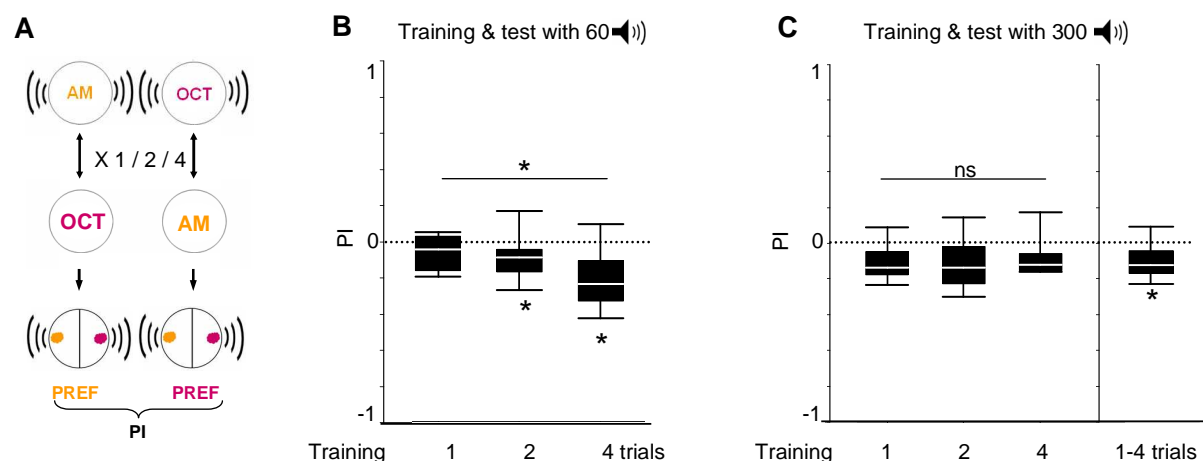

Figure 3.6. Increasing the number of training cycles increases the associative effect. (A) Sketch of the training regimen used, in which the number of training cycles was varied (1, 2, or 4). (B) Using relatively mild punishment (60 buzzes per trial), an increment in the associative effect with an increase of the number of training cycles was observed (dagger for P<0.05 in KW-test, * for P<0.05/3 in OSS-test), whereas more intense punishment (300 buzzes per trial) obscures this dependency (C). The rightmost plot in (C) presents associative performance indices pooled across the number of training cycles, which are significantly different from zero (*, OSS-test: P<0.05). For the underlying preference scores, see Fig.S3.3.

This may reflect that there is an upper limit to the punishing effect of the buzz (at least concerning the particular parameters of the buzz used in this experiment) that cannot be overcome by increasing training cycles, and/or that using too frequent pulses at the moment of testing puts a curb on performance indices: Given that buzzes make the larvae slow down and turn (Fig. 2), using 300 pulses per trial during the test may 'trap' them at their starting position. Indeed, 29 % of the larvae trained and tested with 300 pulses per trial are found in the middle at the moment of scoring, whereas this proportion is only 15 % when only 60 pulses per trial are used. As in odour-taste learning paradigms, one does not need to reckon with such 'trapping' (Schleyer *et al*. 2011), this may partially explain why associative performance indices are smaller in the present paradigm as compared to odour-taste paradigms (*e.g.* Gerber and Hendel 2006).

3.4.6 Testing for effects of the pitch of the buzz

Next, we varied the 'pitch' of the buzz, using 60 buzzes per trial. Specifically, we used buzzes of either 50, 100 or 200 Hz and found that these variations in pitch did not alter training success (Fig. 3.7; KW-test: $P> 0.05$, $H= 1.5$, $df= 2$, $N= 20, 20, 20$; for the pooled data the OSS-test yields: $P< 0.05$, $N= 60$; for the underlying preference scores: Fig. S3.4).
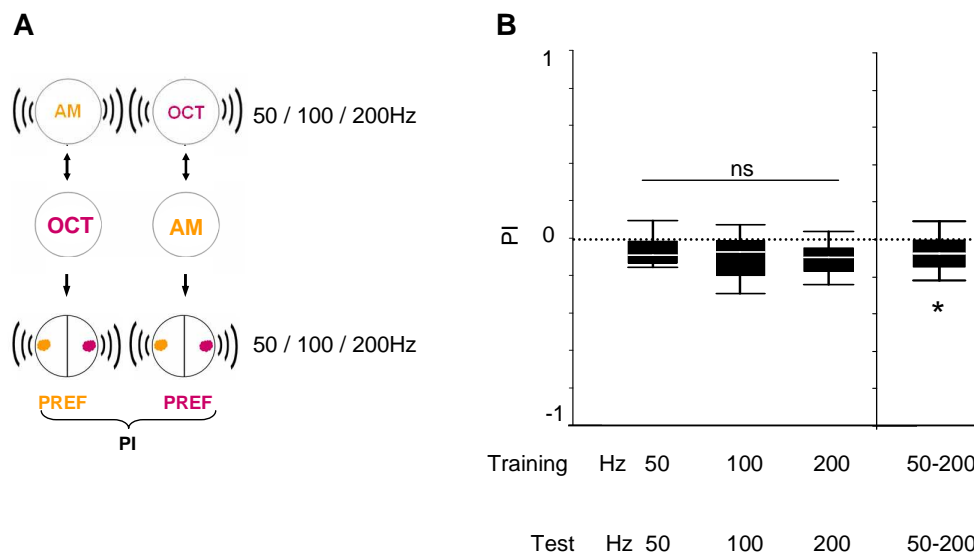

Figure 3.7. Testing for an effect of the pitch of the buzz. (A) Sketch of the experimental regimen, in which the pitch of the buzz was varied (50, 100, or 200 Hz). (B) The associative performance indices are similar irrespective of the pitch used (n.s., KW-test: $P>0.05$); the right-most plot presents the pooled data (*, OSS-test: $P<0.05$). The corresponding preference scores are detailed in Fig. S3.4.

3.4.7 How 'bad' is the buzz?

Given that odour-buzz training endows the odour with the capacity to direct conditioned escape from the buzz during the test, we were wondering whether these odour-buzz memories would also guide escape from other kinds of unpleasant situation. Therefore, we tested the larvae in the presence of either the buzz or aversive tastants (taste; either 4 M NaCl or 0.20 % quinine hemisulfate; at these concentrations, the chemical identity of the tastant is without effect in the present experiments: see Fig. S3.6). Conditioned escape is seen to the same extent for the two kinds of testing situation (Fig. 3.8B, left panel; MWU-test: P>0.05/2, U=1279.5, N= 36, 78) (for the underlying preference scores: Fig. S3.5, left panel).

Interestingly, if the experiment was reversed, that is if larvae were trained with the bad-taste as punishment and were tested either in the presence of that bad-taste, or in the presence of the buzz, conditioned escape occurred to a lesser extent in the presence of the buzz (Fig. 3.8B, right panel; MWU-test: P<0.05/2, U=149, N= 32, 32); indeed, conditioned escape is seen only in the presence of the bad-taste (OSS-test: P< 0.05/2, N= 32), but not in the presence of the buzz (Fig. 3.8B; OSS-test: P>0.05/2, N= 32) (for the underlying preference scores: Fig. S3.5, right panel). How can this asymmetry be understood?

The suggestion of Gerber and Hendel (2006) was that conditioned escape is shown as long as the testing situation is at least as bad as the training reinforcer, whereas no conditioned escape should be observed if the testing situation is less bad than the training reinforcer. Thus, is the buzz less bad than the bad-taste? Indeed, associative performance indices tend to be smaller when the buzz is used for training and testing as compared to when the bad-taste is used for training and testing (left-most versus third plot of Fig. 3.8B; MWU-test: P<0.05, U=353, N= 32, 36). Thus, it seems that the buzz is less strong an aversive reinforcer than the bad-taste, and may not be strong enough to behaviourally activate the association between odour and bad-taste. Alternatively, the bad-taste memory system could be specific in the sense that it is specifically the training-taste that is required for conditioned escape, whereas the buzz memory system may be less specific and can be engaged for conditioned escape by both buzz and bad-taste. However, it would not be trivial to accommodate an aversive memory trace which is specific for the kind of bad stimulus used for punishment. As far as we can see, this would require the existence of (i) separate internal reinforcement systems as well as separate memory traces for buzz and bad-taste, (ii) separate efferent systems to steer conditioned escape which can be modulated by buzz and bad-taste, respectively, and (iii)

selective connections to allow the buzz to modulate only the buzz-related efferences, whereas the bad-taste could engage both kinds of efferences. We believe that, based on the available data, it is more parsimonious to propose that the bad-taste is more strongly punishing than the used buzzes.
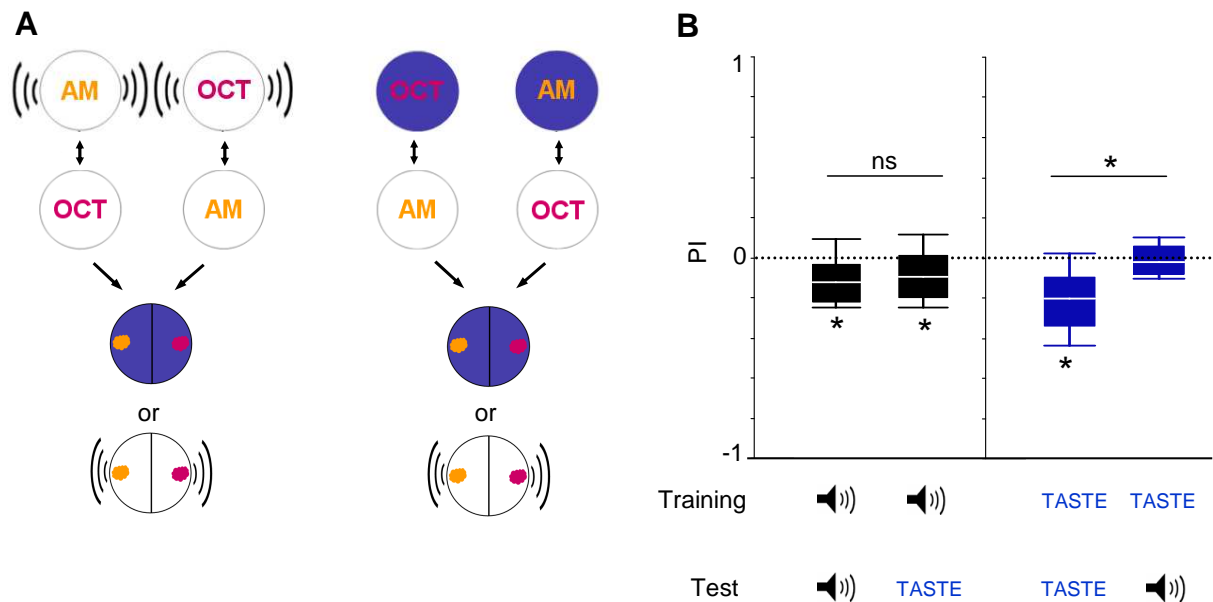

Figure 3.8. How 'bad' is the buzz? A) Sketch of the experimental design. The larvae are trained with 300 buzzes per trial and tested in presence of either these buzzes or a disgusting bitter or salty taste (left panel). In another set of experiments, the larvae are trained with bitter or salty substances as reinforcer and tested in the presence of either the respective taste or the buzz (right panel) (as scores obtained after quinine or salt treatment were not significantly different, the scores were pooled under a "taste" condition unpooled PI and preference scores are displayed in Fig. S3.6). B) The larvae trained with the buzzes show similar associative performances when tested in presence of the buzzes as when tested with taste (n.s., MWU-test: P> 0.05/2; asterisks for P< 0.05/2 in OSS-test). After training with any of the tastants, the larvae show associative performance only when tested on taste; when tested in the presence of buzzes, they do not show associative performance (dagger for MWU-test: P< 0.05/2, * for P< 0.05/2 in OSS-test). For the corresponding preference scores, see Fig. S3.5.

3.5 Discussion

3.5.1 Summary

Here we report that *Drosophila* larvae can associate odours with a mechanosensory disturbance, that is, with substrate-vibration conveyed by a loudspeaker (buzz), as punishment. This model fulfills general expectations for classical conditioning in terms of its parametric dependencies, *i.e.* the increase of associative scores with the number of punishments (Fig. 3.4) and the increase according to the number of training cycles (Fig. 3.6). In contrast, we did not uncover a dependence of the associative process on the pitch of the

buzz in the range between 50 and 200 Hz. However, probing a yet broader range of frequencies could reveal the receiver characteristics regarding the buzz (Fig. 3.7). This may turn out to be interesting in the context of both sensory neurons mediating buzz perception (see below), and the kinds of signal the larvae encounter from animals foraging on their host fruit and/or from parasitoid predators (Dorn *et al*. 1997, Djemai *et al*. 2001). In any event, from the behavioural side, we note that the larvae show an unconditioned escape response towards the buzz (see also Wu *et al*. 2011). Namely, they startle (slow down) and reorient (change direction) (Fig 3.2), a behaviour that is rather robust against experience (Fig. 3.5) and which is observed regardless of its associative predictability (see Results section). Such slow-down-and-turn behaviour has also been observed in response to other types of mechanosensory disturbances such as light touch (Kernan *et al*. 1994), but is qualitatively different, and apparently a level of escalation less, as compared to the 'pain' response when touched by a hot probe (Tracey *et al*. 2003). This pain response involves the product of the *painless* gene, namely a TRP (transient receptor potential) channel expressed in multidendritic neurons (Tracey *et al*. 2003, Hwang *et al*. 2007). Thus, given the distinct nature of unconditioned behaviour towards heat-pain versus the buzz, the buzz signal is probably bypassing the pathway as defined by *painless*-Gal4, and instead is received by tactile and/or proprioceptive sensory neurons (reviewed in Kernan 2007). Indeed, at least the head-turning component of the buzz response is defective upon disrupting the function of chordotonal sensory neurons (Wu *et al*. 2011). It should now be possible to disentangle these sensory pathways in terms of their direct connectivity towards the motor system inducing unconditioned, reflexive behaviour on the one hand, and their connectivity to ascending modulatory circuits to signal reinforcement towards olfactory pathways on the other hand.

3.5.2 Implications regarding the nature of conditioned avoidance

In accordance with what had been suggested by Gerber and Hendel (2006) on the basis of odour-taste learning, conditioned behaviour after odour-buzz learning is not responsive in nature, but rather is driven by its expected outcome. That is, it is not the case that presentation of the learned odour *per se* would trigger conditioned avoidance (Fig. 3.3C, 3.4B). Also, it is not the case that the testing situation *per se* would determine whether conditioned escape is expressed or not (compare left-most versus right-most plot in Fig. 3.8B). Rather, associative performance is based on an interaction of both these aspects. First, the learnt odour activates its memory trace. Second, a comparison is made between the value of this memory trace and

the value of the current situation. Conditioned behaviour then is expressed if the testing situation is at least as bad as what the memory trace suggests. This is in a sense ultra rational, as it is only under these conditions that the larvae can substantially *improve* their situation by expressing avoidance of the punished odour.

Regarding the present analysis, it is noteworthy that the buzz and the bad-taste memories indeed appear to be treated according to their respective level of 'badness': the bad-taste memories are more strongly negative than the buzz memories (left-most versus third plot of Fig. 3.8B), and hence conditioned escape from the buzz-associated odour is seen in the presence of the bad-taste (second plot in Fig. 3.8B), but conditioned escape from the bad taste-associated odour is not seen in the presence of the buzz (right-most plot in Fig. 3.8B). Given that by all likelihood the sensory neurons to mediate bad-taste versus the buzz are distinct, this suggests that both kinds of punishment have access to the same kind of 'bad'-value system to organize conditioned avoidance.

3.5.3 Outlook

Odour-buzz associative learning offers prospect both from the practical point of view, as it lends itself more readily to temporal control of reinforcement and thus to an automation than odour-taste protocols, and because it allows to analyse the neuronal underpinnings on how a relatively simple brain orchestrates memory and behaviour with regard to different kinds of 'bad' events."

Chapter 4

Operant conditioning in larval *Drosophila*?

4.1 Introduction

As detailed in the previous chapter, *Drosophila* larvae can learn about the predictive value of a stimulus, that is, they are capable of classical, Pavlovian conditioning (Pavlov, 1927): Larvae can learn associations between an odour and sugar, or an odour and an aversive tastant stimulus (Scherer *et al* 2003, Hendel *et al* 2005). Notably, this learning does not lead to the formation of a stereotypical response to the odour. Indeed, larvae show learnt behaviour only when needed (Gerber and Hendel 2006; see also Box 1.1 and Chapter 3). These are first hints that larvae actively control their behaviour.

In this chapter, we investigate this question further. We address whether larvae can learn about the consequences of their own behaviour. In this so-called operant conditioning (Skinner 1938), the animal learns that by increasing or decreasing the frequency of a specific behaviour, it will yield specific consequences. For this form of conditioning to happen, the animal needs to strive for a goal, that is, it needs to do something for the appearance or disappearance of an event, and to try out different actions towards that end (Wolf and Heisenberg 1991, Brembs 2003).

For their displacement, larvae perform forward straight crawling by regular peristaltic waves of body-segment contractions (Wang *et al*. 1997). They interrupt this straight crawling from time to time to pause, swing their head in one or many new direction(s), clamp down their mouth hooks on the substrate and crawl further forward (Fig. 4.1). These movements can result in a change in orientation for example towards an odour (Gomez-Marin *et al*. 2010). The pattern of such locomotion is generated centrally (Varnam *et al*. 1996, Suster and Bate 2002), but the particular central pattern generators for each of the before-mentioned behavioural components are unknown. The rate of turns over straight crawling periods depends on Na+ and K+ ion channels (Wang *et al*. 1997, 2002) and on proper functioning of

cholinergic neurons (defined by Cha-Gal4) (Yang *et al.* 2000, Suster *et al.* 2004). Also, sensory input, and especially mechanosensory input, is an important modulator of locomotion (Caldwell *et al.* 2003, Song *et al.* 2007, Cheng *et al.* 2010).
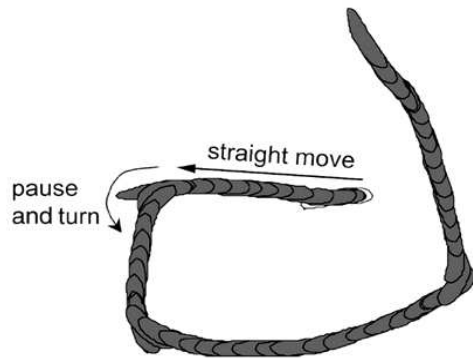

Figure 4.1. Trajectory of a *Drosophila* larva, composed of period of straight crawling accomplished by peristaltic contractions of the body segment, and of periods of pause and turn, initiated by one or sometimes more head swings, towards a new direction. From Suster *et al.*, Genes Brain Behav 2004.

We developed a setup that allowed us to automatically and online detect key components of locomotion of a single larva freely crawling on agarose. We distinguished four behavioural components: forward crawling, turn towards the right of the larva, turn towards its left, and coil. For operant training, we punished the larva for turning on one side, the "punished side", which was alternatedly chosen as right or left for consecutively trained larvae. The punishment was a 200 ms mechanical disturbance ("buzz"), achieved through the activation of a loudspeaker at 100 Hz under the substrate where the larva is crawling. As shown in the case of classical learning (Chapter 3 and Eschbach *et al* 2011b), this buzz can be used as aversive stimulus for *Drosophila* larvae: they show a reflexive, unconditioned response to it by slowing down and turning. Maybe more importantly in the current context, they also show conditioned escape from an odour that was previously associated with such buzzes.

We reasoned that operantly conditioning a larvae such that its turns towards *e.g.* its left are punished could establish two kinds of learnt behavioural strategies during the final test, which would surface under testing conditions of the buzz being either turned off or turned on:

- If the buzz is turned off during the test, they could use a conditioned *avoidance strategy*, where the larvae avoid turning left to <u>not turn on</u> the buzz.
- If the buzz were turned on during the test, they could use a conditioned *escape strategy*, where the larvae turn right to <u>turn off</u> the buzz.

For the present experiments, we opted to turn on the buzz during the test, because our previous findings in classical conditioning (Gerber and Hendel 2006, Schleyer *et al* 2011, Eschbach *et al* 2011b) (Chapter 3) suggest that classically conditioned aversive memories at least are embedded in conditioned escape, rather than conditioned avoidance, behavioural strategies.

4.2 Material and methods

4.2.1 Larvae

We used third-instar feeding stage larvae of the wild-type strain Canton S. The larvae were aged 5 days after egg-laying, kept in mass and raised at 25°C, 60-70% relative humidity and an 14:10 h light:dark cycle.

4.2.2 Setup

The bottom of 145 mm diameter Petri dishes (Sarstedt, Germany) was covered with a thin layer of 1% agarose (Roth, Karlsruhe, Germany) one day prior to the experiments. For each experiment, a Petri dish was placed inside a dark box, on top of a loudspeaker (MC GEE 201847, impedance 8 Ω, diameter 16 cm, acoustic pressure: 89.2 dB/W, power: 150 W RMS), and surrounded by a ring of red LEDs (wavelength: 624 nm). To ensure even dispersion of the light, a 1cm-thick ring of opaque Perspex was inserted between these LEDs and the Petri dish. A webcam placed above allowed recording the arena at 5 frames per second. These frames were analysed online via a tracking software designed in LabVIEW® (Andreas Eckard, University of Würzburg). This software could in turn activate the loudspeaker (Fig. 4.2).

4.2.3 Tracking Software

The software detected a larva crawling on the agarose by luminosity contrast. For each frame, the following parameters were determined (Fig. 4.2, inset):
- position of the centre of gravity in a XY coordinate system;
- orientation of the axis, defined as the longest straight-line fitting into an object, in a 360° coordinate system;
- area of the larva;
- coordinates and area of the bounding box, the smallest rectangular box containing the larva.

These parameters were used to define the action of the larva (Fig. 4.3 & S4.1). To almost immediately detect a change of direction, the current orientation of the larva was compared to its orientation 2 sec before. The software scored a turn any time the differential angle was bigger than a threshold set at 16°, unless the larva was on a coil position. In that latter case, the differential angle calculated by the software was frequently wrong: In the example in Fig. 4.3, a coil towards the left is scored as a turn towards the right. Thus, the orientation readings obtained during a coil, which lasts typically about 2 s, were ignored. These coils were defined from two indicators of the posture of the larva: the "aspect ratio", equal to the ratio of the width over the height of the bounding box, and the "area ratio" calculated by dividing the area occupied by the larva through the whole area of the box. When both indicators approached 1 (respectively 0.8 for the aspect ratio and 0.6 for the area ratio), a coil was detected.
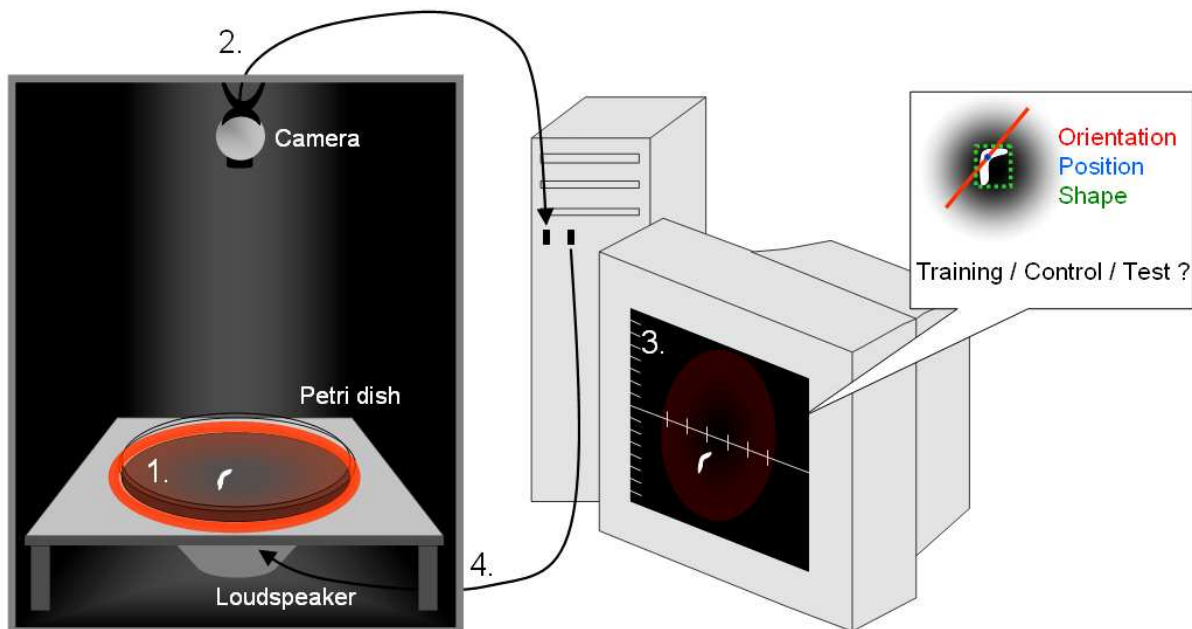
Figure 4.2. Experimental arena. Inside a dark box illuminated with red LEDs, a single larva was free to crawl on a Petri dish filled with Agarose (1.). The Petri dish was fixed on top of a loudspeaker that could deliver mechanosensory disturbances. The behaviour of the larva was recorded by a webcam (2.), analysed online (3.), and, according to training regimen, was used to trigger the onset of the loudspeaker (4.). The inset shows the parameters of the larva determined at each frame by the software: position of the centre of gravity (blue), orientation of the axis (red), area covered by the body, and height and width of the bounding box around the lava (green).

When a turn was detected, no turn was counted for the next frame ("blind period"). For punishment, the loudspeaker was activated during the length of one frame, *i.e.* 200 ms, and played a 100 Hz tone, which triggered the vibration of the Petri dish at a 1,1 m/sec speed.
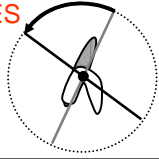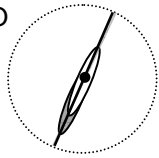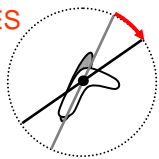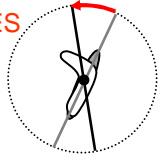
	COIL CRITERIA	TURN CRITERIA	ACTION	BLIND PERIOD DURATION
	Height/Width >0.8 Area ratio >0.6	Differential angle >16°		
	YES	YES	Coil	2 s
	NO	NO	Straight move	
	NO	YES	Turn on the right side	0.2 s
	NO	YES	Turn on the left side	0.2 s

Figure 4.3. Use of the tracking parameters to determine online which action was performed by the larva. Two criteria, the ratio height/width of the bounding box and the ratio of the area of the larva's body over the area of the box, were used to determine from the shape of the larva if it was in a coil position. In that case, the differential angle calculated by the software was frequently wrong, as illustrates the sketch for the angle criteria in the first row. For that reason the axis orientations obtained were ignored for a "blind period" of 2 sec following the detection of a coil. These cases of coil excluded, the current orientation of the body axis was compared to its orientation 2 sec before, allowing an almost immediate detection of orientation change. The differential angle formed by these two axes was compared to a threshold of 16°. A turn was considered any time this differential angle exceeded this threshold. The sign of this angle was used for determining the side of the turn.

4.2.4 Experimental paradigm

A single larva was put in the centre of a Petri dish and tracked for the duration of the experiment. It consisted in a first 3 minutes-phase of either training or control exposure, followed by 1 minute of test (Fig. 4.4). As soon as a larva was not detectable for more than 10 % of the elapsed experimental time, because it was located *e.g.* at the edges, it was discarded.

Trained groups

For the training, a turn towards either the larva´s left or its right is defined as the punished side, and is followed by a buzz (defined as a 200 ms-vibration of the loudspeaker at a frequency of 100 Hz and a speed of displacement of 1.1 m/s). If the larva is still turning towards the punished side after this buzz, a new buzz is triggered. The punishment stops as

soon as the turn is finished, is interrupted by a turn towards the respectively other, safe side, or if a coil is detected. After this training the larvae were tested for one minute, during which the loudspeaker was constantly on. This situation was set to motivate *operant escape* from the buzz by turning towards the previously safe side.

Control groups

To control for effect of the exposure to buzzes, we used a "yoked" control group. Specifically, we exposed a group of larvae to a pseudo-random sequence of buzzes for 3 minutes, created on the basis of the sequences of buzzes experienced by three trained larvae. This procedure ensured a similarly timed exposure to buzzes for both trained and control larvae, the yoked group, however, not having a chance to link its behaviour to the venue of the punishment. Therefore, the turns of the yoked group were either spontaneous or are reflexive, unconditioned responses to a buzz. As for the trained larvae, a one-minute test was performed while a continuous buzz was played. For the calculation, a turning side was arbitrarily attributed as the "punished side" for the control larvae.

Evaluation of the performance

For analysis, we examined the latency until the very first turn towards the previously punished and the latency until the first turn towards the previously safe side during test. We also compared the frequency of turns towards either side during training and test and calculated for that purpose an "operant score" for every minute based on the respective numbers of turns (#):

$$\text{Operant score} = (\#safe - \#punished) / (\#all).$$

This score is comprised between -1 and 1, with positive values indicating preferential turning towards the safe side and zero indicating no preference.

4.2.5 Statistics

One-sample sign tests (OSS-tests) were used to compare values to zero. Pair-wise comparisons of paired samples were made using Wilcoxon signed-rank tests. When multiple comparisons were made within one experiment, we applied a Bonferroni correction; that is, the criterion of significance (0.05) was adjusted by dividing it by the number of comparisons performed, such that the experiment-wide error remains below 5 %. All statistical tests were performed with Statistica 7.1. Data are presented as box-whisker plots, with the middle bold

line indicating the median, the box boundaries indicating the lower and the upper quartile, and the whiskers the 10 % and the 90 % percentile.

ACTION	PUNISHMENT VENUE			
	Right turns are punished	Left turns are punished	Control exposure	Test
Coil			◀))) (0.2 s)	◀))) (continuous)
Straight move			◀))) (0.2 s)	◀))) (continuous)
Turn on the right side	◀))) (0.2 s)		◀))) (0.2 s)	◀))) (continuous)
Turn on the left side		◀))) (0.2 s)	◀))) (0.2 s)	◀))) (continuous)

Figure 4.4. Consequences of the actions of the larvae according to the group they belonged to – punished for right-turns, punished for left-turns or yoked control – and according to the phase of the experiment – either training/control exposure, or test. During the first 3 minutes of training, the two first groups were punished by a short buzz (0.2 s ◀) whenever turning towards the punished side. By contrast, the larvae of the yoked control group received a same total amount of buzzes during the 3 minutes of exposure as the trained groups, but irrespective of their actions, such that the punishment for these animals could happen either during a coil, during straight walk, or during a turn. During the one minute of test, the three groups received a continuous buzz (continuous ◀) to reveal their escape strategy from this buzz.

4.3 Results

4.3.1 Unconditioned response or operant behaviour?

As shown in Chapter 3, larvae respond to a buzz by slowing down and increasing their turning propensity. Our operant training, however, led to a particular situation in which the venue of the punishment itself was triggered by a turn: If for example the left side was assigned as the punished side, the larva 'by definition' had just turned towards its left when the buzz is administered. The larva´s reflexive, unconditioned response towards that buzz

might then be a turn towards its right, *i.e.* towards the respective other side. Indeed, examining the orientation change that followed the first buzz experienced by the larvae during training, we observed that they had a strong tendency to direct themselves towards the safe side just after the buzz (Fig. 4.5). Larvae that were punished for turning towards the right, changed their direction towards the left side during the seconds following the buzz (OSS-test: $p < 0.05/10$), and correspondingly the larvae that were punished for turning towards the left moved towards the right (OSS-test: $p < 0.05/10$ for sec 1 to sec 6 after the buzz). As these larvae experienced the association between buzz and their behaviour for the very first time, this observation could not be due to any operantly learned behaviour. Therefore, concerning the turns observed during the rest of the training phase, one cannot unambiguously distinguish a simple unconditioned response from an operantly learned behaviour.

Therefore, also with regard to the behaviour of the larvae during test, one needs to reckon with the contribution of reflexive, unconditioned components of behaviour as they could be carried-over from the last buzz of training. As can be seen in Figure 4.5 for the first buzz experienced during training, the reflexive, unconditioned response to the buzz was finished after 8 s (Fig. 4.5). Thus, in order to 'distil' operantly learned behaviour, *i.e.* to make sure that test behaviour is clear of any confound by reflexive, unconditioned responses propensities lingering from the last training buzz, we excluded those larvae that received their last training buzz less than 8 s before the start of the test (*i.e.* between the 172nd and 180th sec. of experimental time); we could thus analyse 78 out of the 118 trained larvae, and 24 out of the 40 control larvae. As can be seen in Figure 4.6, this criterion indeed effectively ensures that turning propensities of the considered larvae are back at chance level at the beginning of the test.
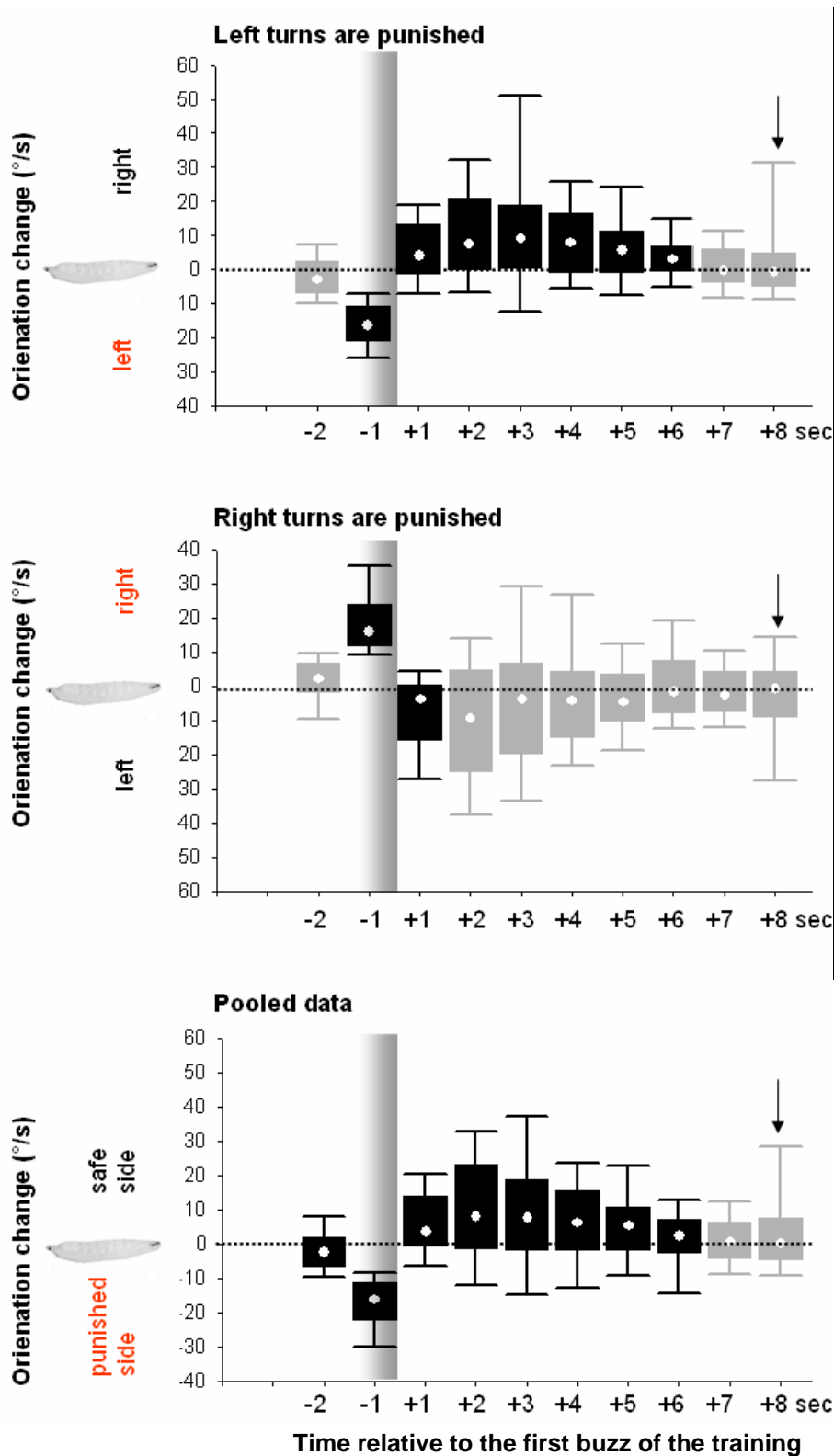
Figure 4.5. Response of the to-be trained larvae to the very first buzz they experience during training. We considered the orientation change in degrees per second. In A is showed the responses of the larvae which were punished for a turn on the left side (N= 60); in B the responses of the larvae punished for a right turn (N= 58); in C the pooled responses of the two groups (N= 118). The vertical shaded line indicates the time point where the first buzz happened. Logically, for the second preceding the buzz, the larvae were turning towards the side that was defined to be the punished side for that larva. For the second following the buzz, they changed their orientation for the respectively opposite side. Black boxes indicate significant differences of p< 0.05/10 from zero (which equal straight move) in OSS-test. It should be noticed that other buzzes might follow the first one depending on the behaviour of the larva.
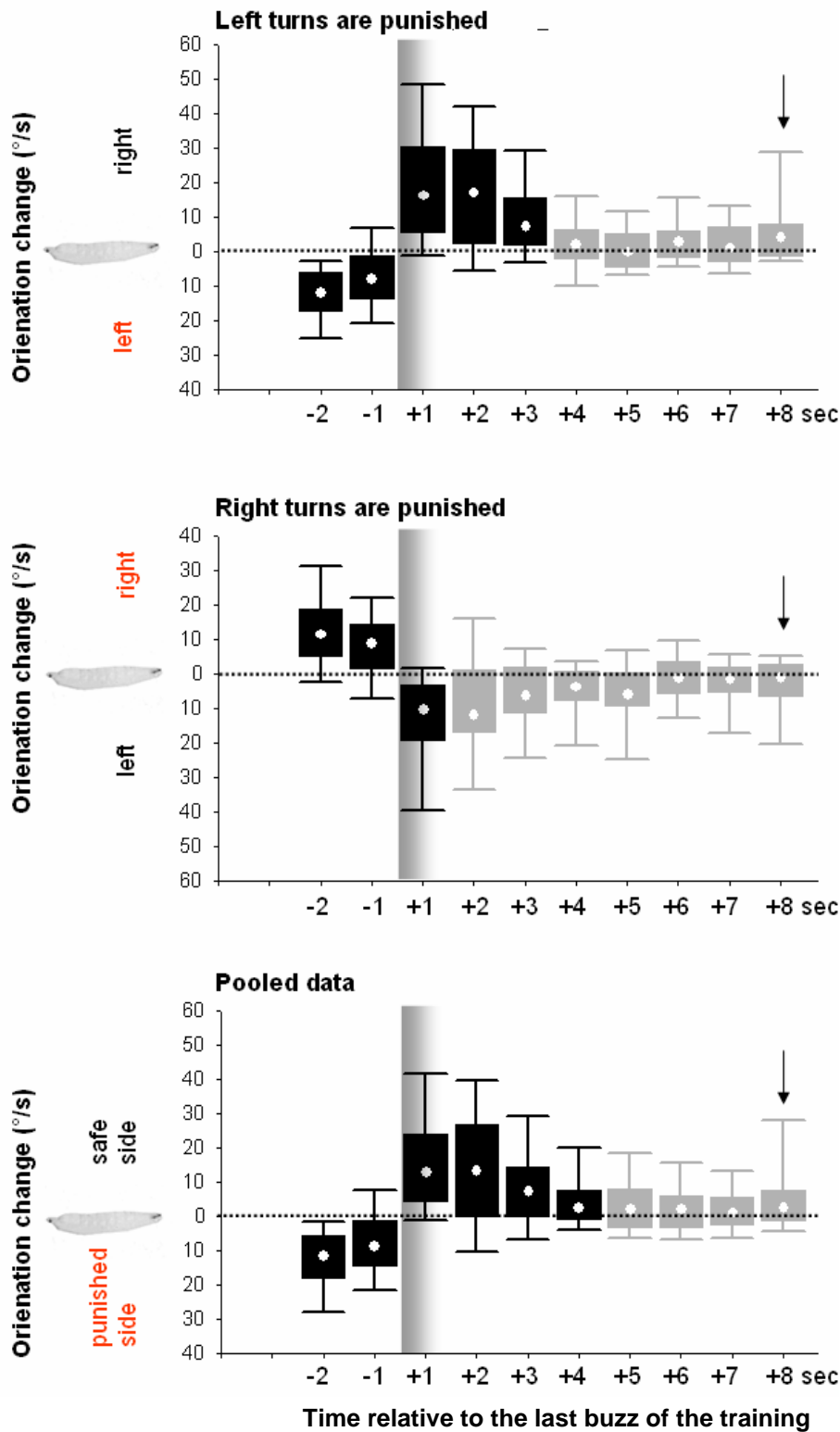
Figure 4.6. Behaviour of the trained larvae to the last buzz experienced during training. Only the data concerning those larvae that were punished more than 8 s before the start of the test are shown (right-turn punished larvae: N=37; left turn punished larvae: N=41). As in Fig. 4.5, the orientation changes of the larvae at the time preceding the last buzz were towards the punished side. We observed a similar reversal of orientation after the buzz as we have observed for the first buzz experienced during training (Fig. 4.5). This behavioural change lasted for one second in the right-turns-punished larvae and for 3 seconds in the left-turns-punished larvae. Thus our elimination criterion of 8 s ensured that the larvae had no lingering reflexive unconditioned turning propensity towards the previously punished side. The calculation of the data, the organisation of the figures and the statistical threshold are the same as for Fig. 4.5.

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4.3.2 Operant conditioning?

For the test behaviour of each larva, we determined the latency to perform its first turn towards the previously punished and the latency to turn towards the previously safe side (Fig. 4.7). In the case of the trained larvae, this delay was shorter for the safe-side turns than for the punishment-side turns (Wilcoxon-test: P= 0.02, T= 1021, N= 78). By contrast, the yoked control group did not show any difference in delay (Wilcoxon-test: P= 0.9, T= 147, N= 24). As unconditioned turning propensities lingering from the last training-buzz do not need to be reckoned with (Fig. 4.6), this shorter latency to turn towards the previously safe side should reflect a learnt operant strategy. Notably, this strategy did not seem to be maintained for a long time (Fig. 4.8). This might reflect a very rapid extinction, which means that the larvae quickly learnt that the learned operant strategy was not effective any longer.
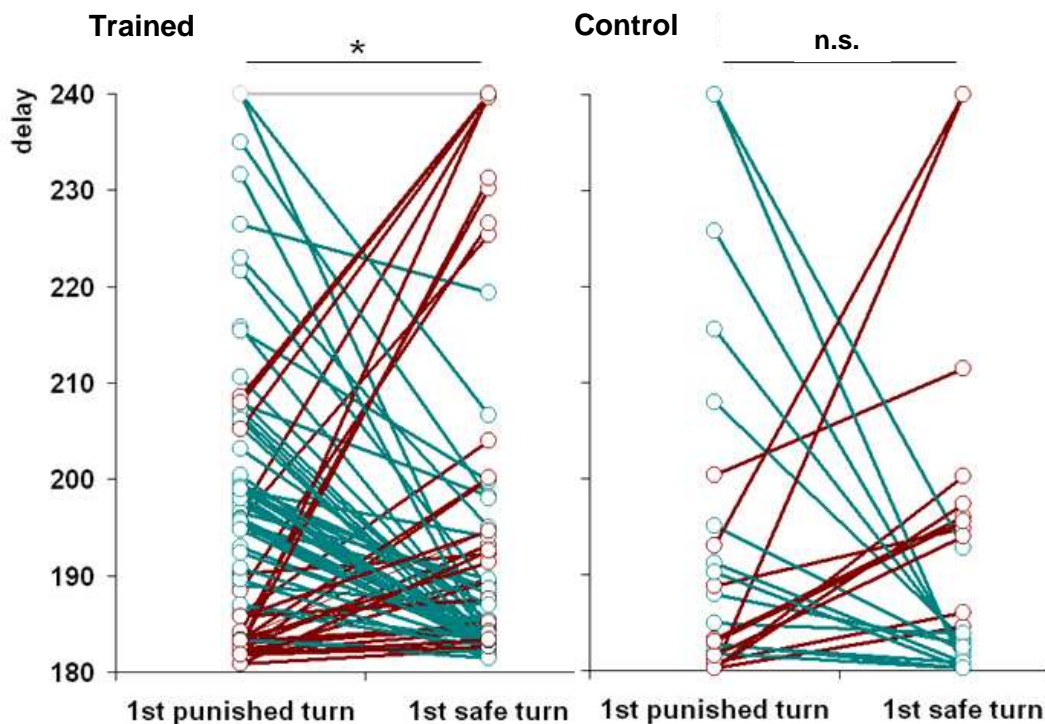
Figure 4.7. Delay to perform the first turn towards the previously punished side and the first turn towards the previously safe side during test, either after training (Trained: N= 78) or in yoked controls (Yoked: N= 24). Of the 78 trained larvae, 47 showed a shorter-latency to turn towards the previously safe side (green), whereas 27 show longer latencies (red). No such difference is observed in the yoked control group. Note that the larvae that might still be responding to the last training-buzz are not considered analysis (see Figure 4.5 and 4.6).
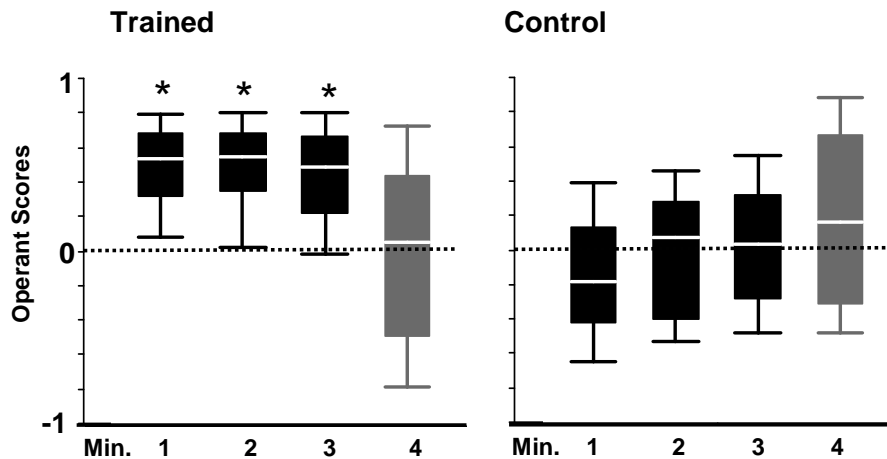
Figure 4.8. Operant scores, taking into account the frequency of punished and safe turns, calculated for trained (left panel) and control larvae (right panel) for each minute of the experiment. During the three first minutes of training, the trained larvae showed clear positive scores, which indicate a strong bias for turning on the safe side (OSS-test: $P < 0.05/4$, $N = 118$). During the test however, this score drop to zero (OSS-test: $P > 0.05/4$, $N = 118$). The control larvae had scores of zero during the whole 3 minutes of control exposure as well as during the test (OSS-test: $P > 0.05/4$, $N = 40$).

4.4 Discussion

We found the first indications of operant conditioning in *Drosophila* larvae. By punishing a larva for turning towards *e.g.* its left, we induced a short-term modification of locomotion such that during the test the delay for the first turn towards its right side was shorter than for the first turn towards its left (Fig. 4.7). As mentioned in the Introduction, our training regimen allowed two kinds of learning to take place:

- Learning that turning towards *e.g.* the left triggers buzz punishment. This would entail a conditioned *avoidance strategy* during the test, where the larvae avoid turning left.
- Learning that turning towards *e.g.* the right turns off the buzz. This should entail a conditioned *escape strategy* during the test, where the larvae turn right to turn off the buzz.

Notably, we had opted to use a testing condition with the buzz continuously on, in order to facilitate the expression of a conditioned escape strategy rather than a conditioned avoidance strategy.

We note that in classical conditioning using pairings of odours with the buzz, a minimum of 60 buzzes per 5 min-training trial (*i.e.* 12 buzzes/ min) and a minimum of 2 training cycles (*i.e.* a total of 120 buzzes) was necessary to see significant effects of such

classical conditioning (see Fig.s 3.4, 3.6). By comparison, during our 3 min-operant training, the larvae experienced only between 18 and 68 buzzes (respective 10th and 90th percentile of the trained group; median of 38.5 buzzes, corresponding to 12.8 buzzes/ min); in a classical conditioning experiment, such low numbers of buzzes would likely not yield detectable learning effects. If indeed the total amount of punishments received during operant training were limiting the observed operant conditioning effects, training the larvae for longer periods and/or with stronger punishment might increase operant learning success. Certainly, however, the current data do not allow the conclusion that operant conditioning effects were weaker than classical conditioning effects.

In any event, these first results of operant learning in larval *Drosophila* make us wonder about its neural and genetics correlates. A first issue concerns the representation of *e.g.* "turning left". In adult *Drosophila*, the central pattern generators responsible for left-right bargaining is arguably located in the central complex (Strauss and Heisenberg 1993, Strauss 2002). Interestingly, mutants presenting central complex phenotypes as adults show locomotion defects as larvae, too (Varnam *et al*. 1996), although the central complex as a discernable brain structure is apparently absent during larval stages until the late third-instar (Young and Amstrong 2010). Thus, one might suspect that the developmental stage of the larva would be critical for allowing operant conditioning. A second issue would be the molecular nature and the cellular site of the memory trace underlying operantly conditioned behaviour. Recently, it was suggested that cellular short-term memory of locomotion can be formed at the level of the motoneurons via genetic effects on the Na+/K+ pump (Pulver and Griffith 2010). This is interesting as the formation of operant memory, although not yet well understood molecularly, may rely on changes in intrinsic neuronal excitability rather than on synaptic plasticity (reviewed in Brembs 2003 and 2011, Mozzachiodi and Byrne 2010).

As I have précised it in the general introduction of my thesis, operant conditioning involves some necessary abilities, certainly in a higher proportion than classical learning: It requests high activeness in the search for the cause of the reinforcer, copy of the motor program and detection of the coincidence between the action and its outcome. Thus, if *Drosophila* larvae emerge as capable of anticipating the consequences of their own actions, this discovery would allow a greater comprehension of operant learning, not only at the level of its mechanisms, but also concerning its implications in terms of the evolutive advantages conferred to such a simple animal as is the *Drosophila* larva.

Chapter 5

Locomotion in Larvae: effect of Rhodiola, SYN, SAP and substrates

5.1 Introduction

When moving, *Drosophila* larvae have to decide where to go. For their displacements they perform forward straight crawling interrupted from time to time by pauses, during which they swing their head and choose a new direction to crawl further (Wang *et al*. 1997, see also Fig. 4.1). Locomotion is thought to be elicited centrally by the rhythmic activation of "central pattern generators", neuronal circuits which command parts of the motor system (von Holst and Mittelstaedt 1950, Varnam *et al*. 1996, Suster and Bate 2002). Also, mechanosensory and especially proprioceptive afferences are necessary for proper coordination of locomotion (Suster and Bate 2002, Caldwell *et al*. 2003, Ainsley *et al*. 2003, Xu *et al*. 2004, Song *et al*. 2007, Cheng *et al*. 2010, Wu *et al*. 2011).

Furthermore, odours generally attract larval *Drosophila*, largely by organizing turning behaviour (Cobb 1999, Gomez-Marin *et al*. 2010); modulations of turning behaviour also underlie the repulsion by as well as the escape from light (Busto *et al*. 1999, Scantlebury *et al*. 2007, Rodriguez Moncalvo and Campos 2009). In both these cases, *changes* in the stimulus input are critical for the respective behavioural adaptations. Furthermore, when crawling on a nutritive substrate, the locomotory behaviour of the larvae is accompanied with feeding (Sokolowski and Hansell 1992, Ruiz-Dubreuil *et al*. 1996).

Normal locomotion thus involves complex mechanisms and interactions between sensory, cognitive and motor systems, and some aspects of locomotion have found a neurogenetic explanation: the protein scribbler (Yang *et al*. 2000) whose expression in the cha-Gal4 neurons is involved in normal turning behaviour (Suster *et al*. 2004), the post-synaptic protein Amphiphysin (Leventis *et al*. 2001), mitochondria associated-proteins expressed by the genes *tamas* (Iyengar *et al*. 1999) and *slowmo* (Carhan *et al*. 2004), Na$^+$ and K$^+$ channel subunits, respectively expressed by the genes *paralytic* and *hyperkinetic* (Wang *et*

al. 1997, Wang *et al*. 2002). Larval locomotion is also modulated by the biogenic amines tyramine and octopamine (Saraswati *et al*. 2004, Fox *et al*. 2006).

In this chapter, we examine the effect of some genetic and environmental manipulations on locomotion behaviour:

- Effect of food supplementation with Rhodiola

- Deletions of two synaptic proteins, Syn and SAP47

- Effect of the taste of the substrate the larvae are crawling on

5.2 Material and Methods

5.2.1 Larvae

All larvae used were raised in mass culture at 25°C, 60-70% humidity and a 8:16 dark/light cycle. At the age of 5 days after egg laying (third-instar feeding stage) larvae were gently washed in distilled water and individually?. The genotypes of the strains used are mentioned along the Results sections.

5.2.2 Setup

The setup has been described in detail in Chapter 4 (see Fig. 4.2). Briefly, it is composed of a dark box containing the arena to house a Petri dish in which 1% agarose (electrophoresis grade, Roth, Germany), plus in the cases mentioned along the Results section also either of various tastants, has been added and allowed to harden for one day. The arena is surrounded by red LEDs. For the first experiment, the diameter of the Petri dishes (Sarstedt, Germany) was of 145 mm, it was of 92 mm for the second and the third experiments.

A camera positioned on top of the arena recorded the position of the larva every 200 msec for offline analyses. Details concerning each particular experiment are given in the Results section.

5.2.3 Tracking software

The data were analysed with a software based on LabVIEW®, designed for this setup (Andreas Eckart, University of Würzburg). The larva was detected by brightness contrast. For each frame the following parameters are extracted from the detected larva:

- its **position**, given by the x and y coordinates of its centre of gravity,

- its **orientation**, given by the orientation of its axis in a fixed 360° coordinate system,

- the position and size of the bounding box, a rectangle that surrounds the larva,

- its area, total surface covered by the larva.

For each frame, the current position and orientation of the larva were compared to the ones obtained the frame before. The difference in position gave the **distance** covered by the larva between these two frames, that is during 200 ms. The difference in orientation of body axis gave the **angle change** in ° in 200 ms. Since there are two possible angles when two axes are compared (one smaller than 90° and one larger), and as visual inspection confirmed that larvae cannot make turns > 90° in 200 msec, we always used the smaller of these two possible angles for analyses. Furthermore, the sign of the angle change was determined so that negative values meant a move towards the larva´s left, and positive values a move towards its right.

All these parameters were initialised as 0 and then determined every frame. In case of problem of detection of the larva (appr. 10% of the time), the values were replaced by 0, and were excluded for further analyses. As soon as a given larva disappeared to the edges of the arena for more than 10% of the respectively elapsed experimental time, it was discarded.

The analysis of locomotion considered the following characteristics:

- the **speed** (mm/s), equal to the sum of the frame to frame-distances for a whole minute, divided by 60.

- the **angular speed** (°/s), as the sum of the absolute values of the frame to frame-angle changes over a whole minute, divided by 60.

- the **total area visited** (% of the whole arena), calculated from the pixels covered by the trajectory of the larva along the experiment.

5.2.4 Statistical evaluation

Further analysis and statistical evaluation of the data obtained by the software was done with Excel and Statistica (Statsoft, Hamburg, Germany). We use a One Sample Sign test (OSS-test) to compare the scores obtained to zero; a Kruskal-Wallis ANOVA (KW-test) for comparison between several groups, and a Mann-Whitney test (MWU-test) to compare two groups. We apply Bonferroni correction in case of multiple comparisons. Data are presented as box plots with the middle line showing the median and box boundaries and whiskers the 25%/ 75% and 10%/ 90% quantiles, respectively.

5.3 Results

5.3.1. Effect of food supplementation with Rhodiola

The roots of *Rhodiola rosea* are used in folk medicine for their various physiological effects (reviewed in Kelly 2001). In *Drosophila melanogaster*; supplementation of food medium with *Rhodiola rosea* roots increases life span (Jafari *et al.* 2007). Recently such a treatment has also been shown to improve associative performance scores of *Drosophila* larvae in a classical conditioning paradigm associating odour and sugar (Fig. 5.1, from Lushchak *et al.* in preparation). As locomotion is required during such tests of classical conditioning, this increase in the performance might conceivably be due to alterations in locomotion, rather than an improvement of associative function *per se*. In order to exclude that possibility, we investigated locomotion of larvae treated with *Rhodiola* food supplementation.
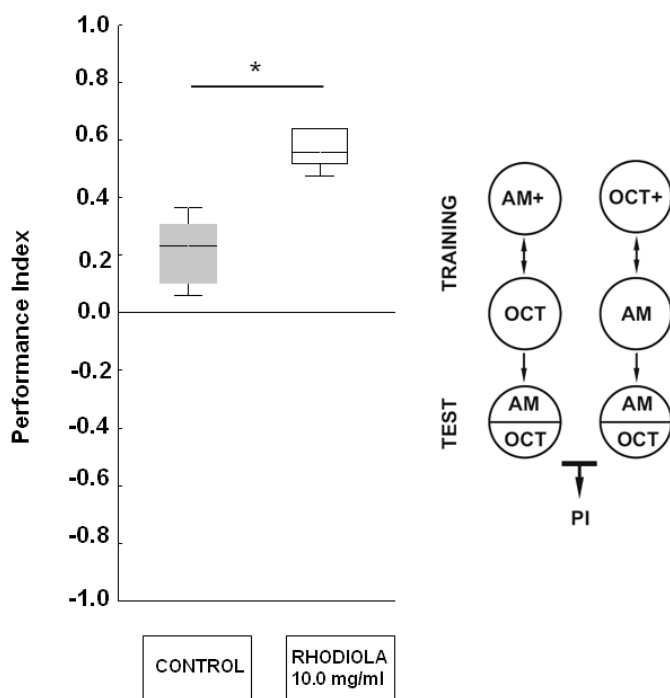
Figure 5.1. Performance Indices of larvae reared on standard food (CONTROL) and of larvae reared with 10.0 mg/ml RHODIOLA added to their food. The performance index measures associative function by comparing the distribution of larvae between the two odours AM (n-amylacetate) and OCT (1-octanol) after either AM was rewarded and OCT was not (AM+/ OCT), or after the reciprocal training regimen (AM/ OCT+); the inset figure illustrates this experimental procedure. Please note that in half of the cases we started training with AM+ or OCT+ as indicated; for the other half of the cases, we started training with OCT or AM. The performances of RHODIOLA-treated larvae differ from the CONTROL in a Mann-Whithney U-test at *P*< 0.05. Figure modified from Lushchak *et al.* in preparation.

We used larvae of the wild-type Canton S strain. Control larvae were raised on standard medium. For RHODIOLA-treated larvae we added 10 mg powder of dried *Rhodiola rosea* roots per ml standard medium. The experimenters were blind concerning food treatment. Individual larvae of each of the groups were left crawling on pure agarose and we estimated their speed and turning propensity.

We did not find significant differences comparing speed (Figure 5.2A, MWU-test: U= 403, P= 0.49, N= 30, 30) or angular speed (Figure 5.2B, MWU-test: U= 395, P= 0.42, N= 30, 30) between control and RHODIOLA-treated larvae, arguing against an effect of RHODIOLA-treatment on the motor abilities of *Drosophila* larvae. As an effect on sensory faculties involved in such associative learning has been previously excluded as well (Lushchak *et al.*, in preparation), the improvement in performance scores in the classical conditioning paradigm after RHODIOLA food supplementation can thus be specifically attributed to increased associative function.
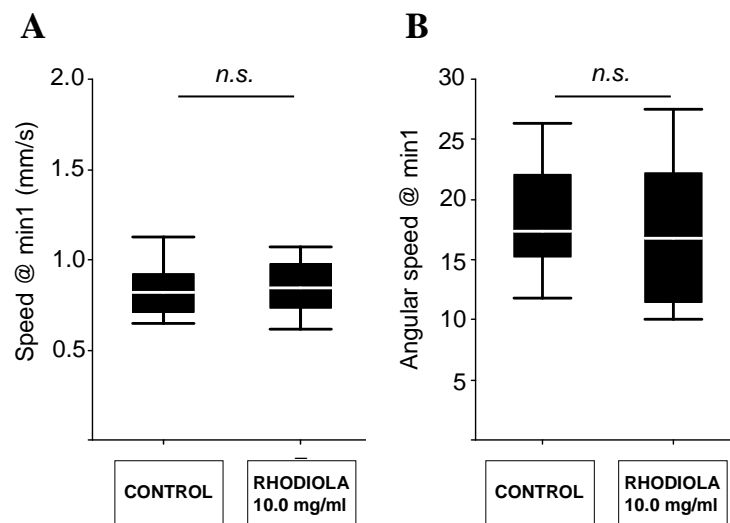
Figure 5.2. Speed (mm/s) and angular speed (°/s) of larvae crawling on agarose for one minute, and reared on standard food (CONTROL) or food supplemented with 10.0 mg/ml of RHODIOLA.

5.3.2 Implication of the synaptic proteins Synapsins and SAP47 in the locomotion

Synapsins are abundant brain phosphoproteins, which are thought to maintain the reserve pool of synaptic vesicles and to mediate the regulated mobilization of reserve pool vesicles towards the readily-releasable pool of synaptic vesicles (Akbergenova and Bykhovskaia 2007, Evergren *et al.* 2007). The Synapsin protein-null mutant Syn^{97CS} has been obtained by a P-element jump-out mutagenesis with a deletion of *ca.* 1.4 kb, which eliminates parts of the presumed promoter region and half of the first known exon (Godenschwege *et al.* 2004, Fig. 5.3A from Michels *et al.* 2005). In adult *Drosophila*, this mutation does not obviously affect synapse functioning but does impair complex behaviours such as the optomotor response, ethanol tolerance, walking, flight (Godenschwege *et al.* 2004) as well as learning and memory to a relatively mild extent (Godenschwege *et al.* 2004, Knapek *et al.*

2010). In larvae, it induces a 50 % defect in the associative performance scores in the larval odour-sugar associative learning (Michels *et al*. 2005, Michels *et al*. 2011). Testing them for their preference towards sugar as well as towards the odours employed, however, indicated that the locomotion of the mutants larvae was globally intact, at least with regard to those faculties needed for the learning task.

Similarly, SAP47 is a highly conserved Synapse Associated Protein in *Drosophila* with a molecular weight of 47 kDa and is localized in synaptic terminals (Funk *et al*. 2004). The *SAP47^{156}* null mutant has been gained by P-element mediated jump-out mutagenesis and shows a *ca.* 1.7 kb long deletion in the *Sap47* locus (Funk *et al*. 2004, Fig. 5.3B from Saumweber *et al*. 2011b). Adult *SAP47^{156}* null mutant flies are viable and fertile and show no obvious phenotype (Funk *et al*. 2004). *Drosophila* larvae carrying this mutation show however a comparable learning defect as the *Syn^{97CS}* mutants in larval odour- sugar learning paradigm (Saumweber *et al*. 2011b), with unimpaired task-relevant sensory and motor faculties.

A double mutant, carrying null mutations for both Synapsin and SAP47 is in the process of characterization in terms of its associative abilities, in order to see whether the respectively partial phenotypes upon lack of Synapsin and SAP47 are additive.

To characterize locomotion in some more detail, we determined speed and turning propensity of larvae which lack Synapsin, SAP47, or both types of protein. Corresponding wild type controls were obtained by extensive outcrossing the mutants with the Canton S-wild type: *CS^{NF}* was the control strain for *SAP47^{156}*, *CS^{45}* the control for *syn^{97CS}* and *CS^{V}* the control for *SAP47^{156}/syn^{97CS}*. These strains were provided by Jennifer Bretzger (University of Würzburg) who verified the absence of the respective proteins in the mutants by a western blot using adult flies' heads, prior and after this study (pers. comm. from J. Bretzger).

The behaviour of individual *Drosophila* larvae crawling on pure agarose Petri dishes was analysed at the first and third minutes of the test. We examined the speed and the turning propensity. Experimenters were blind with respect to the strains studied; those were decoded only after the experiments. The results are presented in Figure 5.4.
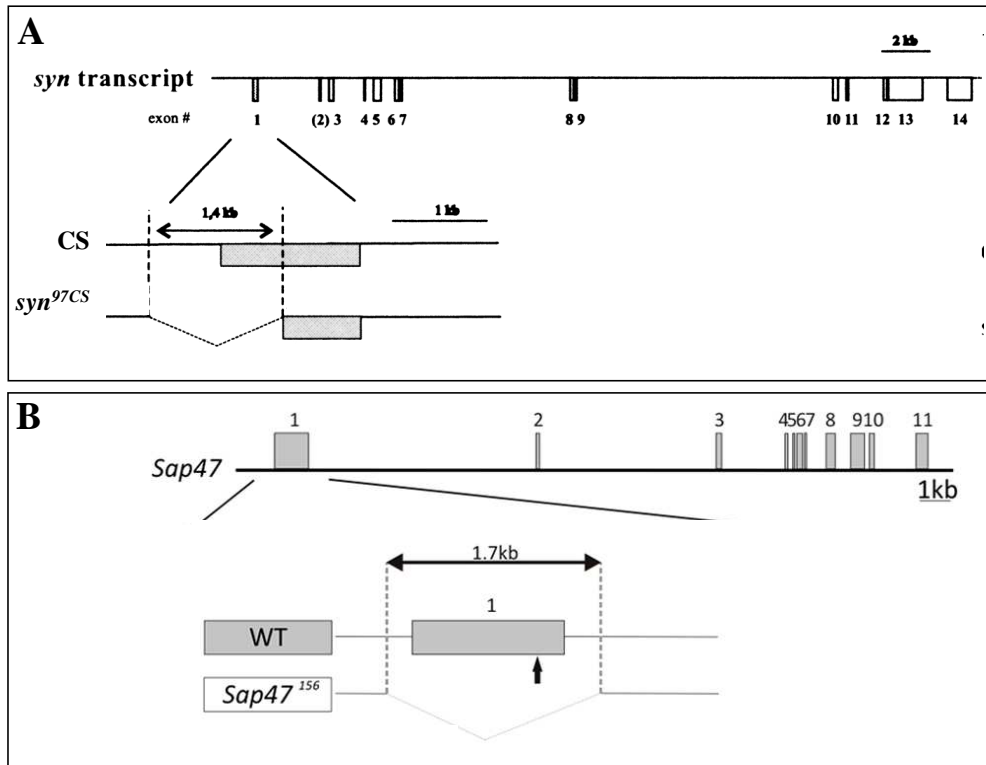
Figure 5.3. Genomic organization of the *Drosophila synapsin* and *SAP47* locus. Boxes represent exons, coding sequences are in black. A) Syn^{97CS} carries a 1.4-kb deletion spanning parts of the regulatory sequence and half of the first exon of the *syn* gene. From Michels *et al*. 2005. B) Genomic structure of the *SAP47* gene of *Drosophila melanogaster*. The deletion in *SAP47^{156}* is shown at higher magnification. The arrow below the first exon indicates the translation start. From Saumweber *et al*. 2011b.

We found a general increase in speed (Wilcoxon-test: P< 0.05/6, N= 27 for each strain) and a general decrease in the angular speed (Wilcoxon-test, P< 0.05/6, N= 27 for each strain) between the first and the third minute of crawling. Comparing each mutant strain with its corresponding wild type, we did not find any difference in speed, neither at the first (MW-test: U= 354, 350.5, 294.5, P>0.05/6 for comparison between respectively *SAP47^{156}*, *Syn^{97}* and *SAP47^{156}/Syn^{97}* and their control), nor at the third minute of crawling (MW-test: U= 347, 282, 235, P>0.05/6 for comparison between respectively *SAP47^{156}*, *Syn^{97}* and *SAP47^{156}/Syn^{97}* and their control). Comparably, the angular speed did not differ between any mutants and their respective wild type strains at the first minute of crawling (MW-test: U= 303, 229.5, 302.5, P>0.05/6 for comparison between respectively *SAP47^{156}*, *Syn^{97}* and *SAP47^{156}/Syn^{97}* and their control). We found however that the *SAP^{156}* mutant had a higher propensity to turn than its wild-type *CS^{NF}*, at least at the third minute (MW-test: U= 201.5, P= 0.004), while no difference was seen between *Syn^{97}* or *SAP47^{156}/Syn^{97}* mutants and their control (MW-test: U= 340.5, 247, P> 0.05/6, respectively).

Thus, we did not find any motor function related-phenotype in larval Synapsin mutant Syn^{97}, unlike what has been found in adult mutant (Godenschwege *et al.* 2004). We found a slight difference in turning propensity in the $SAP47^{156}$ null mutant for SAP47. The fact that we did not find this increase in angular change in the double mutant might indicate either that the motor phenotype observed in the SAP47 mutant is not a genuine effect of the deletion of the protein, that the additional deletion of the Synapsin protein counteracts this effect, or that this is a false-positive result- despite our attempts to correct significance levels by a Bonferroni-correction.
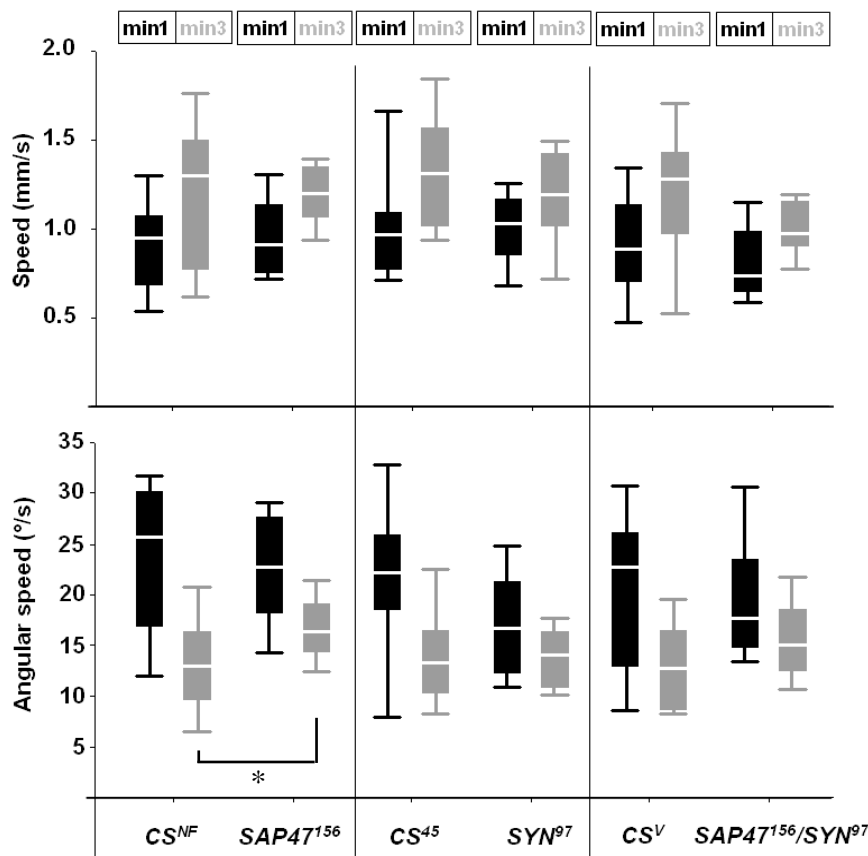
Figure 5.4. Locomotion in $SAP47^{156}$, its wild-type control CS^{NF}, syn^{97CS}, its control CS^{45}, $SAP47^{156}/syn^{97CS}$, and its control CS^{V} larvae. The speed (mm/s) and angular speed (°/s) of larvae are measured during the first and the third minutes of observation. Difference in shading indicates a significant difference in a Wilcoxon-test ($P < 0.05/6$) between minute 1 and 3 in a given genotype, the asterisk indicates a difference in a MWU-test between a mutant an its control at a given time ($P < 0.05/6$).

5.3.3 Effect of the taste of the substrate

For such a notorious feeder as the *Drosophila* larva is, taste is a key information for deciding what to do. The organisation of gustatory receptors and primary neurons seems to

follow a rule of roughly binary classification between good/eatable and bad/uneatable substances (reviewed in Gerber and Stocker 2007, Gerber *et al*. 2009). Also behaviourally, as defined by feeding or choice behaviour in larvae, a gustatory substance could be classified in terms of attractive versus repulsive. Sugar, salt, and bitter processing are well studied in that regard.

More precisely, fructose feeding and preference are higher when mixed with agarose at an intermediate concentration (0.1 to 1M) and decreases at higher concentration (Schipansky *et al*. 2008). Aversion for quinine is already present at very low concentration (less than 5. 10^{-4}M) and increases in a dose dependent way (El Keredy *et al*. in preparation). Choice behaviour regarding NaCl of increasing concentration in agarose shifts from attractive (until 0.1-0.2 M) to repulsive (from 0.3M) (Niewalda *et al*. 2008). Interestingly, for each of the tastants considered, the reinforcing value in classical conditioning seems to be less sensitive than the nutritive value: appetitive learning keeps increasing at high concentration (2M) of fructose (Schipansky *et al*. 2008), and the concentration dependent-shift from appetitive to aversive learning using NaCl as reinforcer is observed 'later' in the concentration scale (0.5-1M) than is the shift observed in naïve preference (Niewalda *et al*. 2008, Russel *et al*. 2011).

I here describe the first step that we made in order to understand how taste preference is behaviourally achieved in *Drosophila* larvae, in term of modification in speed or turning propensity. For statistical analysis we compared observations made in 'PURE' conditions to the other conditions. We used larvae of the wild-type Canton S strain and performed two types of experiment during which individual larvae were observed for 5 minutes:

- In a first experiment, we compared parameters of locomotion of individual larvae crawling on either pure agarose substrate (PURE), or agarose either mixed with 2M Fructose (FRU, purity 99%, Roth, Karlsruhe, Germany), 0.25M NaCl (low SALT, purity 99.5 %, Roth), 1.5M NaCl (high SALT), or 0.20% Quinine (QUI, purity 92 %, Sigma-Aldrich, Munich, Germany) (Fig. 5.5A, N= 74 for each condition).

- In a second experiment, we studied whether previous experience of the larvae affects the parameters of the locomotion on PURE substrate. To do so, we first allowed the larvae to crawl for one minute on a Petri dish with PURE, FRU, low SALT, high SALT or QUI, and

then followed their locomotion on PURE substrate for five minutes (Fig. 5.6A, N= 25 for each condition).

Regarding the first experiment, the substrate the larvae were crawling on indeed induced differences in locomotion of the larvae at the first min of observation, in terms of both speed (Fig. 5.5B, KW-test: H= 39, df= 4, P< 0.05/2) and angular speed (Fig. 5.5C, KW-test: H= 35, df= 4, P< 0.05/2). Larvae crawling on PURE moved faster than larvae crawling on FRU, low or high SALT (Fig. 5.5B, MWU-test: P< 0.05/10) and had a higher turning propensity than larvae crawling on FRU or high SALT (Fig. 5.5C, MWU-test: P< 0.05/10).
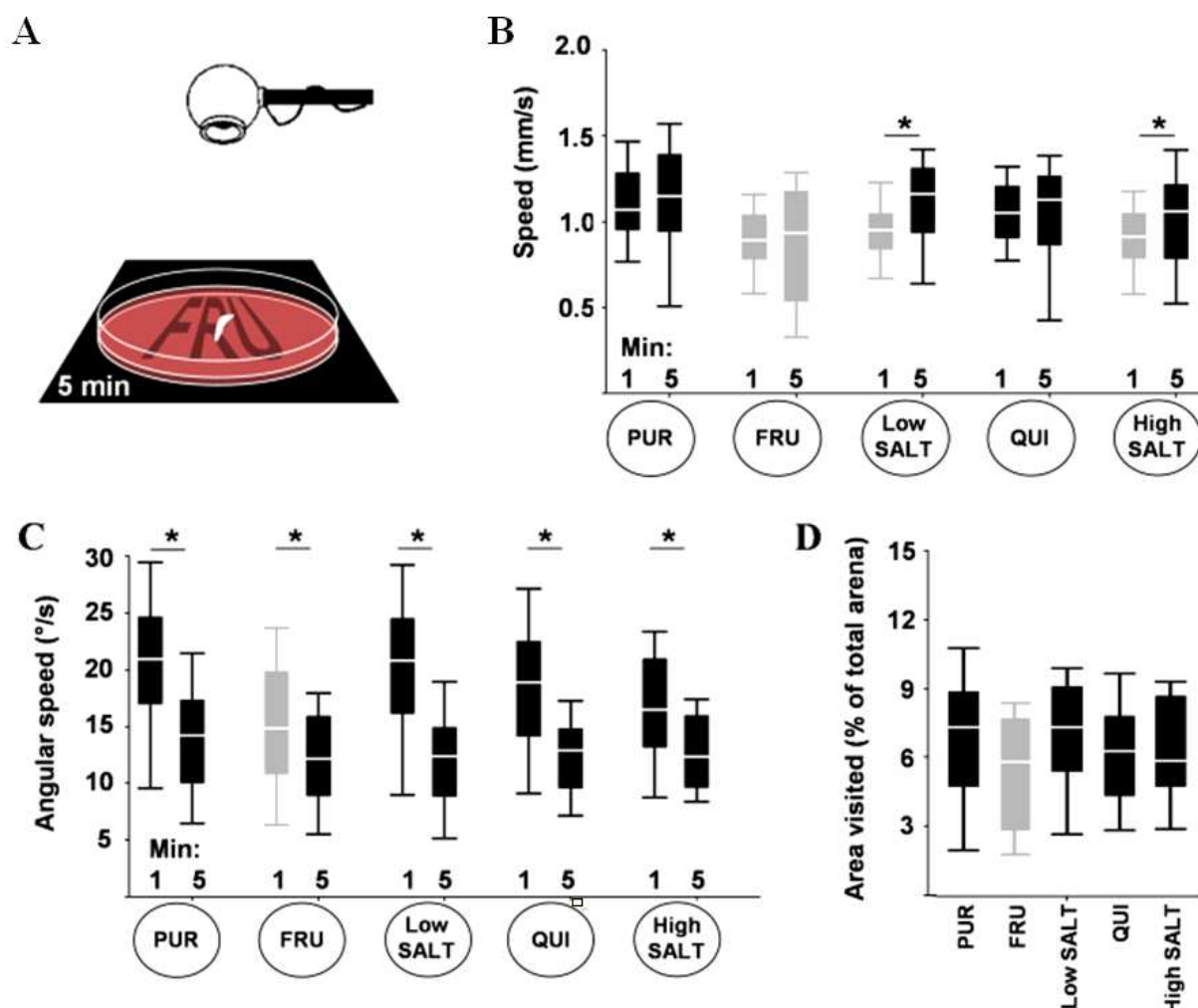
Figure 5.5. Crawling behaviour of larvae crawling on agarose either PURe either mixed with 2M FRUctose, 0.25M NaCl (low SALT), 0.20% QUInine or 1.5M NaCl (high SALT) for 5 min, as depicted in the sketch A. In B, we measured the speed in mm/s during the first and the fifth minute of crawling; in C the angular speed in °/s. In D are shown the percentages of the arena visited during the whole 5 min by the respective groups. The asterisks indicate a significant difference (P< 0.05/5) in a Wilcoxon-test in the parameters in a given group between the two time points considered. The grey boxes are the boxes which differ significantly (P< 0.05/4) from the PUR group at the given minute in a MWU-test.

At the fifth minute, differences in speed largely faded (Fig. 5.5B, KW-test: H= 19.3, df= 4, P< 0.05/2); only the larvae crawling on FRU were still slower than the ones crawling on PURE (MWU-test: P< 0.05/5). Differences in angular speed are no more detectable (Fig. 5.5C, KW-test: H= 7.2, df= 4, P> 0.05/2) as all groups of larvae showed a generally lower propensity to turn (Fig. 5.5C, Wilcoxon-test: P< 0.05/5 for all groups) than they had during the first minute. As a result, the larvae crawling on the FRU explored less of the total arena (5.8 %) than did the larvae crawling on PUR (7.5 %) (Fig. 5.5D, KW-test: H= 15, df= 4, P< 0.05, MWU-test: P< 0.05/10 for FRU group).

For the second experiment, all larvae were observed while they were crawling on PURE. However, prior to that, the larvae experienced for one minute either PURE agarose, or agarose mixed with one of the tastants (Fig. 5.6A). This one minute-experience did not significantly influence the locomotion behaviour of the larvae, neither at the first nor at the fifth minute of crawling: larvae from all groups moved equally fast (Fig 5.6B, KW-test for the respective first and fifth minutes: H= 4.5 and 5, df= 4, P> 0.05/2), turned equally much (Fig. 5.6C, KW-test for the respective considered minutes: H= 3.5 and 4, df= 4, P> 0.05/2) and uniformly explored in total ca. 6.6 % of the total arena during the five minute (Fig. 5.6D, KW-test: H= 4.8, df= 4, P> 0.05).

These experiments show that larval locomotion can be directly modified due to the substrates the individuals are crawling on (Fig. 5.5). Some of the observations, especially the fact that larvae are less moving in presence of FRU than in presence of PUR, could at least partially explain how a larva 'prefer' one substrate over the other in a preference test, as they would quit the PURE side faster and would be more likely to stay on the FRU side (Schipansky *et al*. 2008). Such a mechanism, however, could not explain the observed aversion of QUI (El Keredy *et al*. in preparation) or high SALT (Niewalda *et al*. 2008), which would thus use other mechanisms, maybe ones requiring the transition between PURE and the respective tastant side in the choice assay. Indeed, orientation towards an odour relies on the comparison of directional information through active sampling of the olfactory environment (Cobb 1999, Louis *et al*. 2008, Gomez-Marin *et al*. 2010). Genetic manipulations, which let only a single unilateral olfactory neuron functional, did alter but not delete the chemotaxis skills of *Drosophila* larvae (Louis *et al*. 2008), indicating that a short-term temporal comparison between sensory inputs does happen at certain time points, certainly when the larvae are swinging their head in different directions before turning.
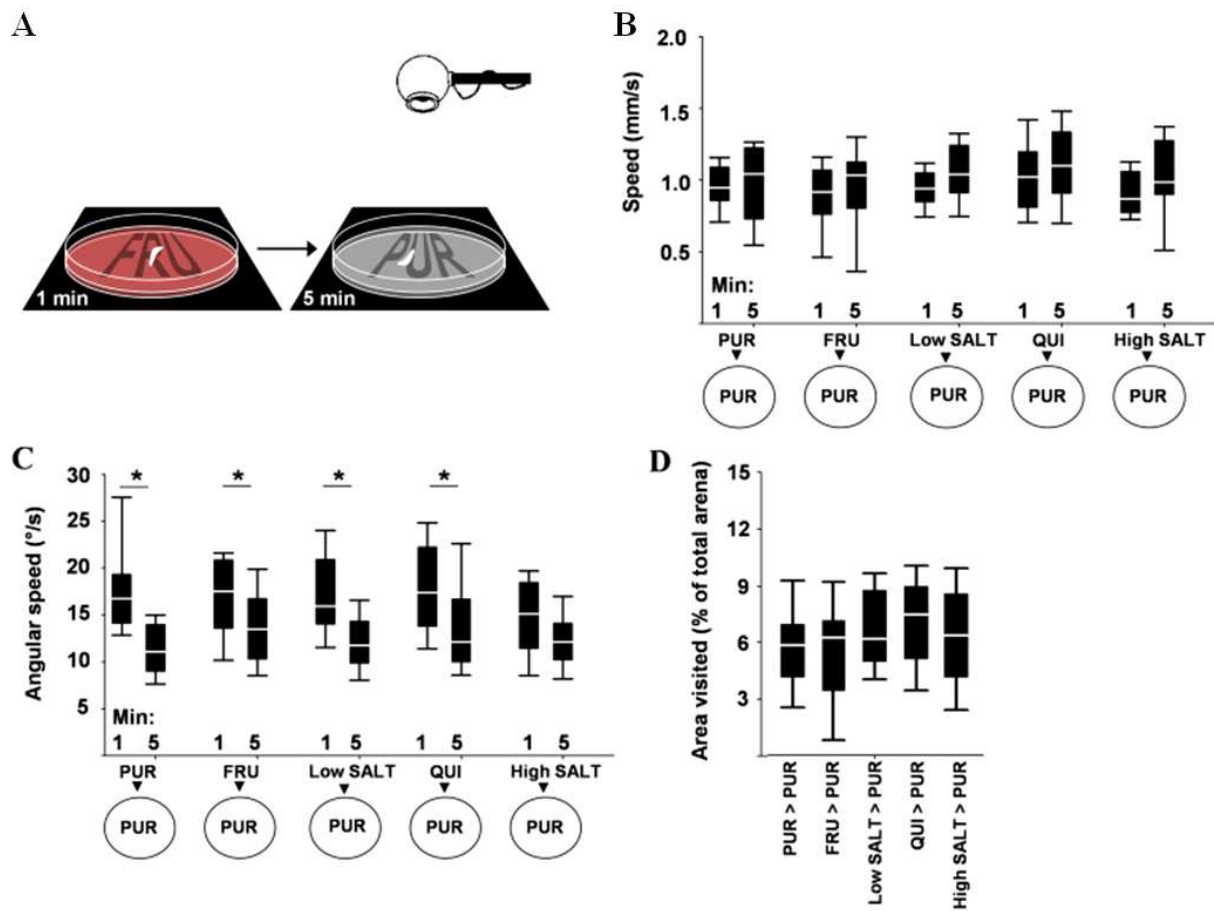
Figure 5.6. Crawling behaviour of larvae on PURe agarose during 5 min after having crawled for one min on either PURE, FRU, low SALT, QUI or high SALT substrates, as depicted in A. The locomotion is described in terms of speed (B) and angular speed (C) at the first and the fifth min of crawling, and the percentage of the total arena visited during the five min (D). Other details are the same as in Fig 5.5.

Such mechanisms may also take place during taste-orientation, that is, the larva might regularly sample the gustatory state of the substrate and change its direction and/or speed when it is going worse. Interestingly, our results suggest that these kinds of process may in particular be relevant with regard to 'bad' taste.

We also note that the larvae on the putatively nutritive substrates (FRU, low and high SALT) were moving slower than the larvae that were crawling on non-nutritive substrates (QUI or PURE). One could interpret this observation as the result of feeding behaviour taking place while crawling and which would slow down translational movement. This however is not supported by data obtained on ingestion. Indeed, at our considered concentration, FRU (2M fructose) or high SALT (1.5M NaCl) substrates are *less* ingested than PURE (Schipanski *et al*. 2008, Niewalda *et al*. 2008).

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Last, the motor features exhibited by larvae experiencing the different substrates might serve as visible 'signatures' of the presence of each of these substrates to their companion larvae. This would mean that when larvae perceive *e.g.* FRU, they would slow down. It would be interesting to see whether some of those characteristics are conserved when the larvae are tested for their odour preference after odour-fructose learning.

Chapter 6

Conclusions and perspectives

In this thesis I have used behavioural approaches to study psychological phenomena in adult and larval *Drosophila*, especially in the context of associative learning.

I first described our study on olfactory perception in adult *Drosophila* (Chapter 2) which revealed that a mixture formed by two odours is perceived as similar to both the elements composing it, with no cue of mixture-specific interaction in its processing. Methodologically we insisted on the importance of equal behavioural potency of the odours used in order to avoid misinterpreting olfactory perception as (even) more complex than it seems to be.

The following chapters focused on larval behaviour. We showed the reinforcing effect of a new aversive stimulus: mechanical disturbance via loudspeaker-induced vibration, in classical conditioning (Chapter 3) (and maybe also in operant conditioning: Chapter 4). This enriches the short library of stimuli meaningful to this animal and should generate progresses in the neurobiology of mechanoperception, classical conditioning and memory. We confirmed that the conditioned behaviour towards an odour is driven by its expected outcome concerning buzz reinforcement. The findings suggest that mechanical and gustatory punishment might share to some extent the same internal reinforcing signals. The odour-buzz associative conditioning presented here allows investigating this question further by genetic techniques. It also offers the possibility to temporally manipulate the venue of the reinforcement and in the longer term to automatise the conditioning protocol.

Regarding operant conditioning, I reported the first results suggesting operant conditioning abilities in *Drosophila* larvae. Future research in that direction will hopefully confirm these findings. The simplicity of the paradigm used to train the larvae operantly, combined with the many genetic possible manipulations of this animal, would then allow detailed investigations into the cellular, molecular and genetic bases of this until now largely mysterious form of learning.

The last part of the thesis described the first steps to characterize the influence of factors that enhance cognitive functions, in the case of *Rhodiola,* or impair it, in the case of *Syn* or *SAP47* null mutations, on the normal behaviour of the larvae (Chapter 5). They allow separating the behavioural manifestations linked to learning abilities from others, and thus pinpointing more precisely the mode of action of the molecules of interest. Also, we described behavioural changes due to presence of food or uneatable substances. This would help defining the link between those substances – either presented alone as we did here, or as a stimulus reinforcing an odour – and locomotion.

To summarize, this thesis probed the range of psychological phenomena which could be studied in *Drosophila* larva: behavioural organisation, associative conditioning, information processing and expectation, a range which could tentatively be extended into operant conditioning. The future will hopefully push these boundaries yet further into attention, decision, etc., and into their respective neurogenetic mechanisms.

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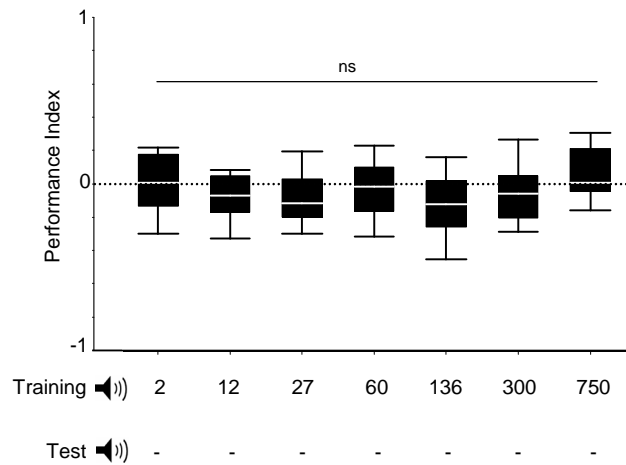
Supplementary material

Figure S3.1. With regard to the experiment displayed in Fig.·3.4 (leftmost plot in B), the associative performance indices of the groups tested in the absence of the buzz are separated according to the number of training cycles. Given that in the absence of the buzz the odour–buzz memories are not behaviourally expressed, scores were statistically indistinguishable from each other (ns, Kruskal–Wallis test: $P > 0.05/2$), and when pooled were not different from chance (see Fig. 3.4B). Sample sizes are from right to left: 22, 25, 25, 29, 25, 25 and 25.
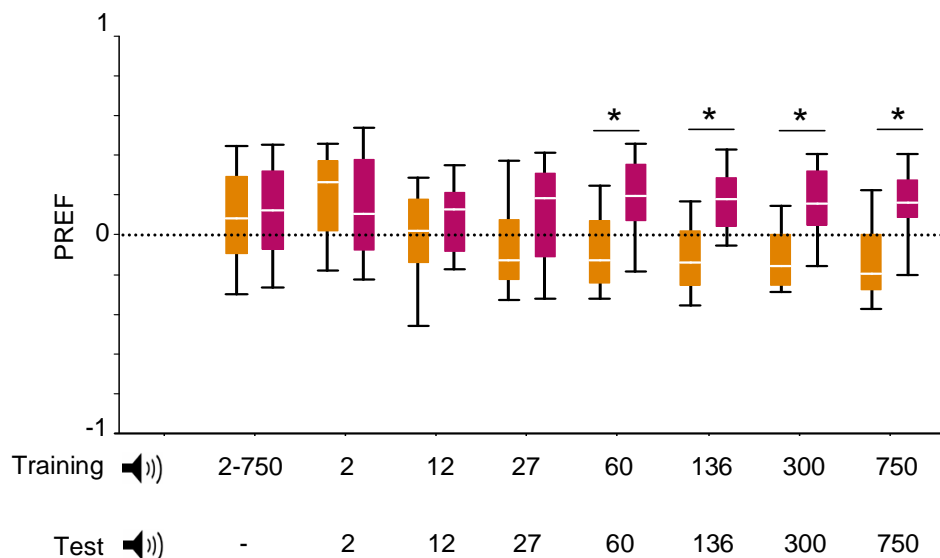

Figure S3.2. Preference scores underlying the associative performance indices displayed in Fig. 3.4B (* $P < 0.05/8$ in Mann–Whitney U-test). Orange boxes: buzz paired with AM; purple boxes: buzz paired with OCT. Positive scores indicate preference for AM; negative scores indicate preference for OCT.
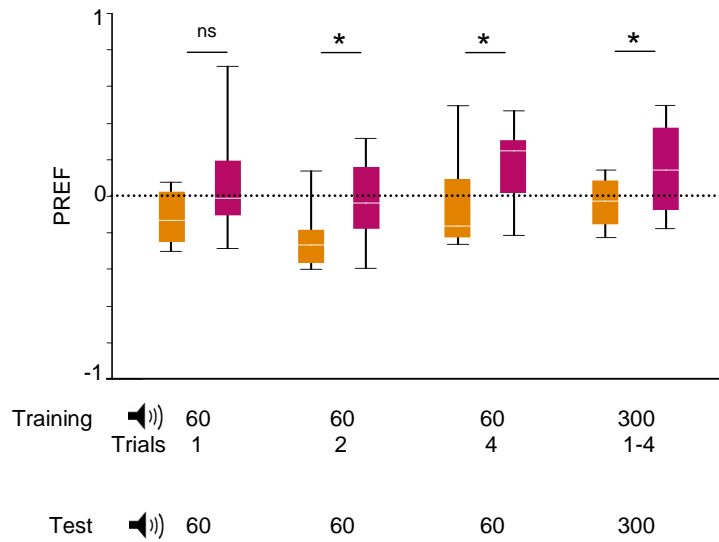
Figure S3.3. Preferences scores underlying the associative performance indices displayed in Fig. 3.6 (* for P< 0.05/4 in MWU-test). The six plots to the left relate to Fig. 3.6B, the two plots to the right relate to the rightmost plot of Fig. 3.6C. The colour code is the same as for Fig. S3.2.
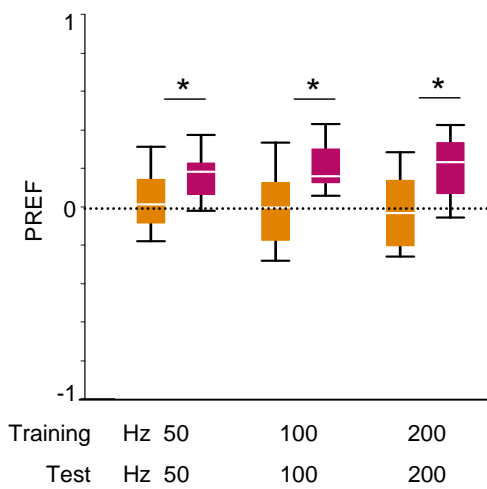

Figure S3.4. Preferences scores underlying the associative performance indices displayed in Fig. 3.7 (* for P< 0.05/3 in MWU-test). The colour code is the same as for Fig. S3.2.
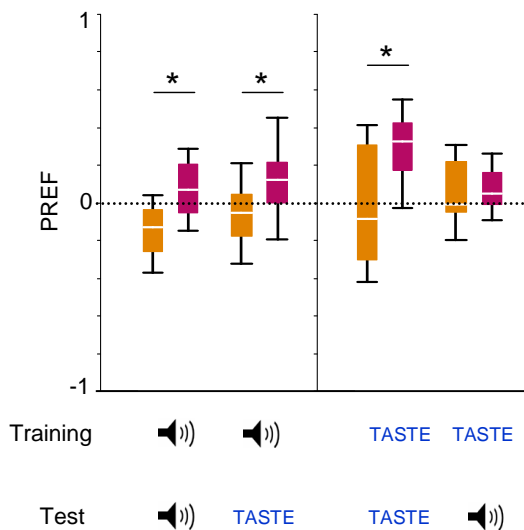

Figure S3.5. Preference scores underlying the associative performance indices displayed in Fig. 3.8 (*P<0.05/2 in Mann–Whitney U-test). The colour code is the same as for Fig. S3.2.
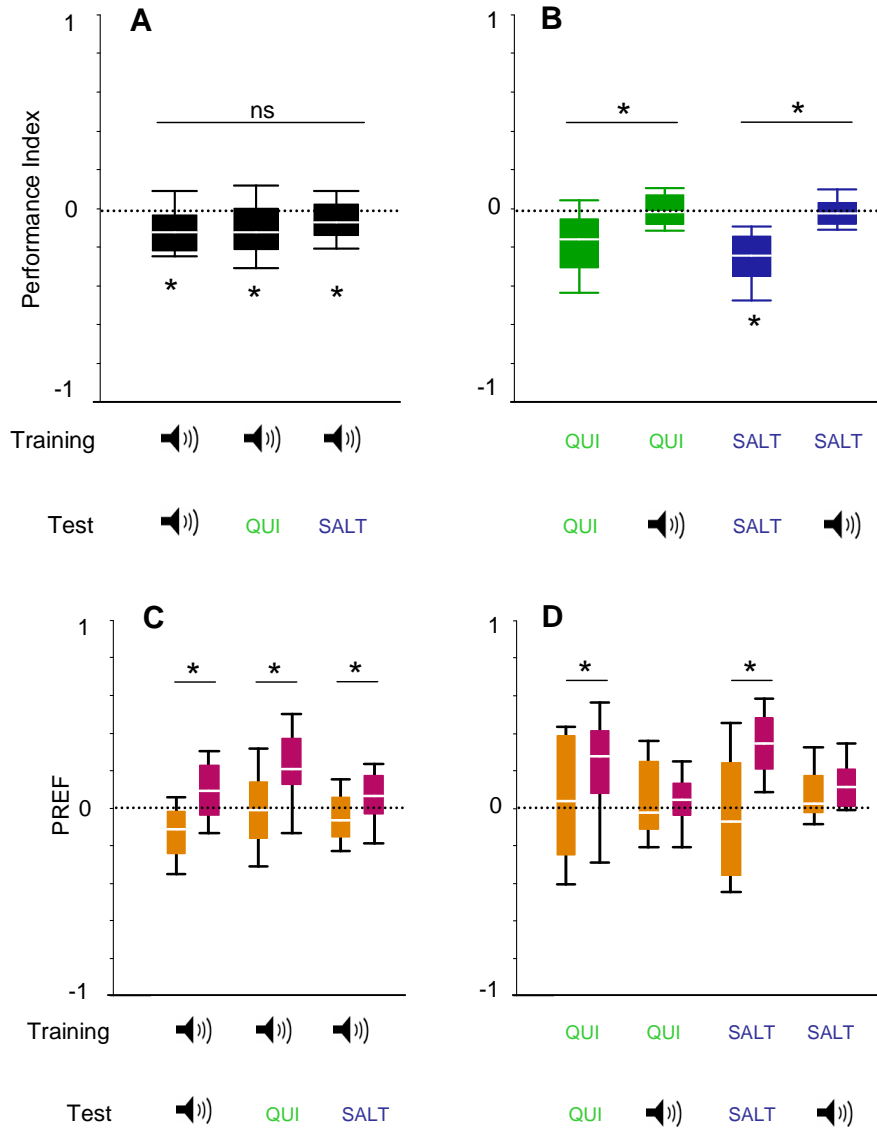
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Figure S3.6. Non-pooled data of the experimental results of Fig.·3.8B. (A) Associative performance indices obtained when larvae are trained with 300 buzzes per trial and tested in the presence of the training buzz (N= 36), or on 0.20% quinine (QUI, N= 36), or on 4 mol l–1 NaCl (SALT, N= 42) (ns: Kruskal–Wallis test, P<0.05; *one-sample sign test: P<0.05/3). (B) Associative performance indices when larvae are trained with QUI or SALT and tested either with the respective training tastant (N= 16, 16) or in the presence of the buzz (N=16, 16) (upper asterisks, Mann–Whitney U-test: P<0.05/2; lower asterisk, one-sample sign test: P<0.05/4). (C,D) The corresponding preference scores for, respectively, A and B (*P<0.05/3 and P<0.05/4, respectively, in Mann–Whitney U-tests). Orange boxes: the aversive stimulus was presented with AM; purple boxes: the aversive stimulus was presented with OCT.
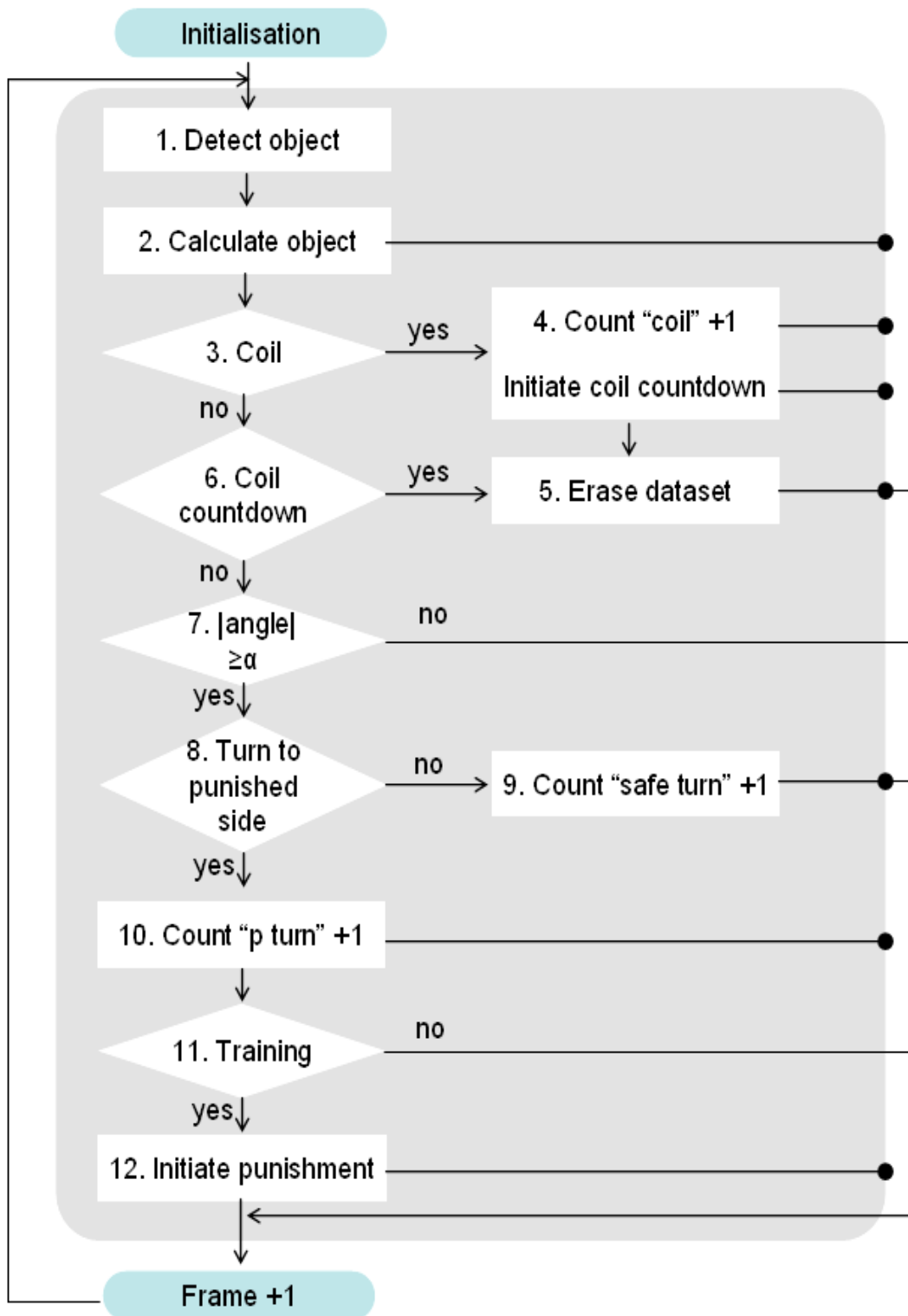
Figure S4.1. Flow chart detailing the modules of the tracking software designed in LabVIEW®. The instructions are run in loop for each frame at 5fps. From the object detected (1), parameters are calculated and saved in an excel file (2); these parameters allows the detection of a probable error in information processing for turn, due to the shape of the larva ("Coil", 3), in which case such a situation is recorded in the excel file (4), a blind period is initiated (5) and is kept for the next 2 sec (6). In the contrary case, the software determines if the larva is turning, *i.e.* if the angle it is forming with the axis 2 s before is more than the threshold angle α (7). If it is the case, the software determines if the turn is on the defined safe or punished sides (8). In the first case, the software counts a "safe turn" (9). In the second case, it counts a "p turn" (10) and during training phase (11), it initiates 0.2s buzz (12).

A combined perceptual, physico-chemical, and imaging approach to 'odour-distances' suggests a categorizing function of the *Drosophila* antennal lobe***

Introduction

"

A flourishing period of research over the past three decades has led to a reasonably detailed picture of how different odours can cause different activity patterns along the olfactory pathway (reviewed in Strausfeld and Hildebrand 1999, Hallem *et al.* 2006, Fiala 2007, Vosshall and Stocker 2007, Gerber *et al.* 2009). In insects, odours are detected by sensory neurons housed within sensillae on the third antennal segment and the maxillary palps. These sensory neurons project to the antennal lobes, the functional equivalent of the olfactory bulb in vertebrates. Each sensory neuron typically expresses one functional *Or* receptor gene, endowing different types of sensory neuron with partially overlapping ligand profiles (Hallem and Carlson 2006, Kreher *et al.* 2008). Those sensory neurons expressing a common *Or* receptor gene then converge onto one glomerulus within the antennal lobe (Couto *et al.* 2005). For different odours, this entails combinatorially different activity patterns of glomeruli (Fiala *et al.* 2002, Ng *et al.* 2002). Within the antennal lobe, local circuits that comprise interneurons and projection neurons shape the olfactory signal (reviewed in Wilson 2008). From the antennal lobe the projection neurons, corresponding to the mitral cells in vertebrates, relay to the lateral horn, a presumed premotor center, as well as to the Kenyon cells of the mushroom body (Marin *et al.* 2002, Wong *et al.* 2002, Murthy *et al.* 2008, Aso *et al.* 2009), which may be viewed as a 'cortical' structure (Tomer *et al.* 2010). Output from the mushroom bodies then projects to presumed premotor areas as well (Ito *et al.* 1998, Tanaka *et al.* 2008, Heimbeck *et al.* 2001). Here we ask at which stage of this pathway neuronal activity patterns correspond to perception in the fly (for a pioneering study in the bee: Guerrieri *et al.* 2005).

We define perception in behavioural terms: If two stimuli are perceived differently, these differences should enable the fly to differentially behave towards them. We first provide such an operationally defined, behavioural account of perceived distance between odours. Then, we ask at which stage along the olfactory pathway a fit is found between odour-evoked activity patterns and the salient features of these behavioural measures of perception.

Results

A behavioural handle on perceived difference

Our approach was to ask whether flies perceive a test odour *as the same* or *as different* from a previously learned olfactory stimulus. Therefore, dose-effect functions of learnability first needed to be determined, such that odour concentrations could be chosen that support equal learnability for all odours (Fig. A.1, 2A). This is important to ensure symmetry of similarity judgements (see Discussion). Also, to keep reasonably clear of task-specific confounds, we used four behavioural tasks (i-iv below) to 'distill' the salient, task-independent perceptual relations between odour pairs. We therefore needed to choose relatively few odours, and decided for those that have in the past been used most frequently in the field (benzaldehyde: B; 3-octanol: O; 4-methylcyclohexanol: M; n-amylacetate: A).

Tasks (i) & (ii). Flies were trained by presenting an odour together with electric shock and then were tested either for their avoidance of that trained odour (Fig. A.2A) or for their avoidance of a novel, not previously experienced odour (Fig. A.2B) (in this as well as in all following tasks, flies were trained and tested only once). When novel odours were used for testing, learning scores were in all cases symmetrical (Fig. A.2B): Scores were equal when *e.g.* 3-octanol (O) was trained and n-amylacetate (A) was tested as compared to the case when A was trained and O was tested (the two right-most plots in Fig. A.2B). We therefore pooled the respective subgroups for further analyses. It turned out that in most cases hardly any learned behaviour was observed towards novel odours, reflecting perceived dis-similarity between trained and tested odour. To quantify this perceptual dissimilarity, we determined a 'Perceptual Distance Score': If training and testing odours are actually identical (perceived distance is zero), we found learning indices as corresponding to the stippled line in Fig. A.3A. We reasoned that to the extent that perception of the test odour deviates from the trained odour (*i.e.* perceived distance between them increases), the smaller learning indices should be found. Thus, the degree to which learning indices were degraded by presenting a non-trained
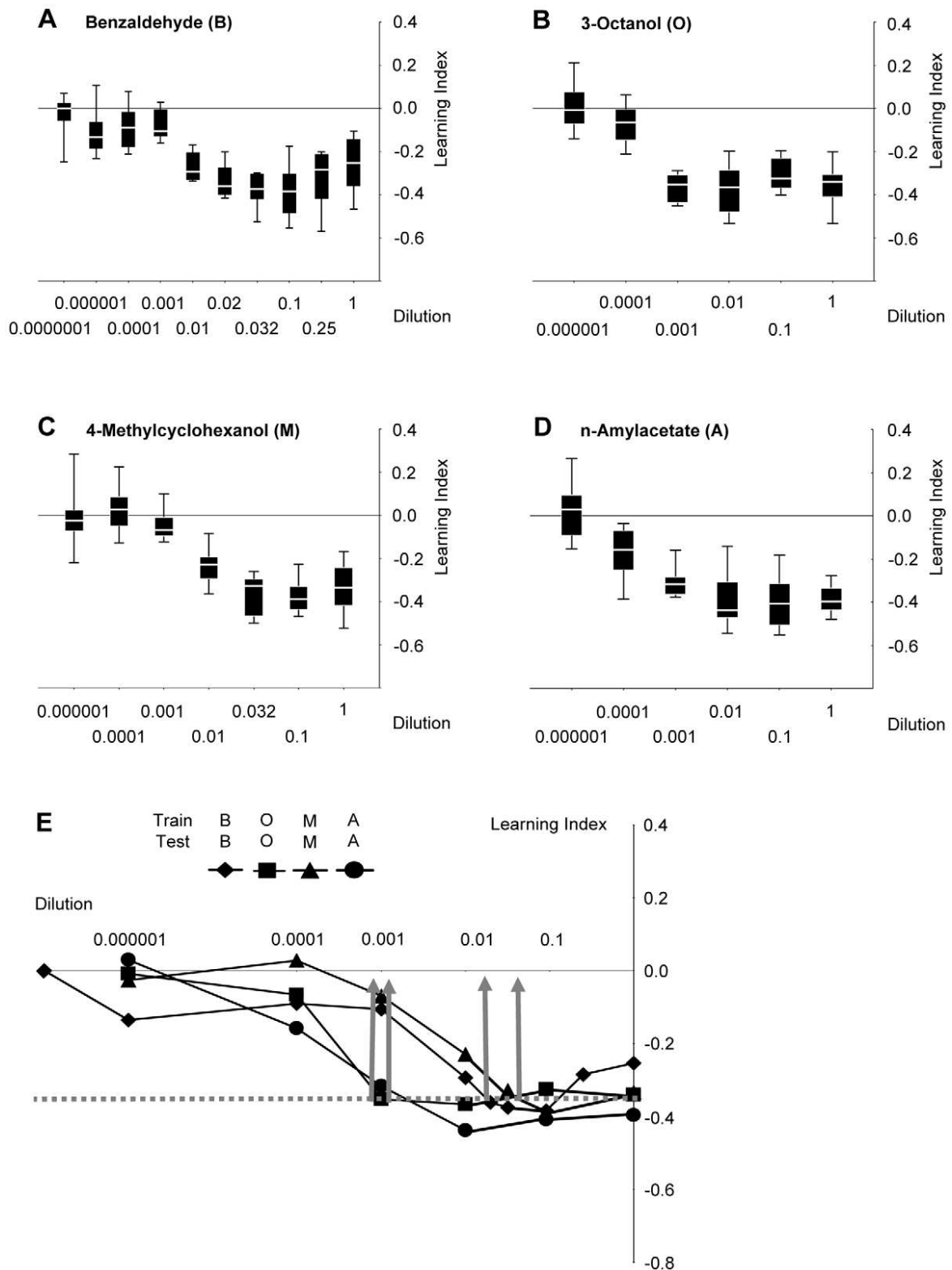
Figure A.1. Adjustment of odour intensity for equal learnability. (**A, B, C, D**) Flies are trained with a given odour at the indicated dilution, and then are tested using that same odour at that same dilution. Sample sizes are for B: 12, 12, 12, 12, 12, 12, 12, 8, 8, 8; for O: 11, 12, 12, 12, 8, 8; for M: 12, 8, 12, 12, 12, 8, 8; for A: 12, 12, 12, 12, 8, 8. Data are presented as box plots (middle line: median; box boundaries and whiskers: 25%/75% and 10%/90% quantiles). (**E**) Median data from (**A, B, C, D**) combined. Note that while asymptotic learning scores do not differ between dilutions, the dilutions at which that asymptote is reached differ between odours across almost two orders of magnitude. Dilutions for further experiments are chosen such that learning indices are the same and, for each kind of odour, have just about reached asymptotic levels (stippled grey line and grey arrows) (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000). For sample sizes, see (**A, B, C, D**).

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odour could be used to estimate perceptual distance scores (arrows in Fig. A.3A). We noted that for training with O or A allowed the respective other odour to elicit the highest learning scores, both (i) when scores were taken immediately (Fig. A.3A, A′) and (ii) when they were taken after an additional retention period of 180 min (Fig. A.3B, B′) (see Fig. A.S1 for the symmetry of the 180-min scores). We interpreted such behaviour as reflecting perceived similarity between these two odours.
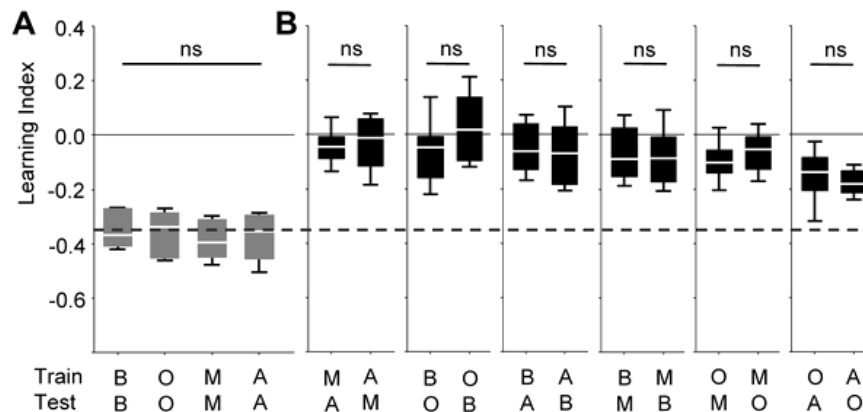

Figure A.2. Symmetry of perceived distance. Learning indices dependent on the combination of TRAINing *versus* TESTing odour (benzaldehyde: B, 3-octanol: O, 4-methylcyclohexanol: M, *n*-amylacetate: A). In (**A**), flies were tested with the trained odour, whereas in (**B**) they were tested with a not previously trained odour. Odour-intensities had been chosen for equal learnability (see Fig. A.1). The stippled line in (**B**) represents the median of the pooled data from (**A**). Learning indices in (**B**) are in all cases symmetrical, in the sense that scores are equal when *e.g.* O was trained and A is tested as compared to when A was trained and O is tested. ns: in (**A**) $P > 0.05$ in a Kruskal-Wallis test, in (**B**) $P > 0.05/6$, Mann-Whitney U-tests using a Bonferroni correction. Sample sizes are from left to right: 11, 12, 11, 11, 16, 16, 16,16, 16, 16, 16, 16, 16, 15, 16, 16.

Task (iii). We trained flies with joint presentations of one odour with electric shock and then tested the flies for their choice between that trained odour versus a novel odour. To the extent that the flies regarded the two odours as different, they should have distributed unequally between them. Thus, in this experiment, perceived distance between the choice-odours should have shown as large learning score (Fig. A.3C, C′). We found that perceived distance was smallest between O and A also in this kind of assay (Fig. A.3C, C′) (see Fig. A.S2 for the symmetry of the scores).

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Figure A.3. (p.36) Concordance of perceived distance across four types of recognition experiment. (**A**) Representing the data from Figure A.2B, pooled for odour pairs. The stippled grey line represents the learning indices that were found when TRAINing and TESTing odour were identical (from Fig. A.2A). To the extent that flies regarded the TESTing odour as different from the TRAINing odour, learning indices should approach zero; thus, the degree to which flies regarded both odours as different can be quantified by the Perceptual Distance Score 1 (red arrows). In (**A′**) these scores were presented normalized to the highest median score thus obtained. Sample sizes are from left to right: 32, 32, 32, 32, 31, 32. (**B**) Same as in (**A**), except that a 180-min break was given between training and test. Sample sizes are from left to right: 24, 24, 24, 24, 24, 24. (**C**) Flies were trained with a given odour, and then were tested for their choice between that trained odour *versus* a novel, not previously trained odour. Thus, if the flies regarded the two TESTing odours as the same, scores should be zero.
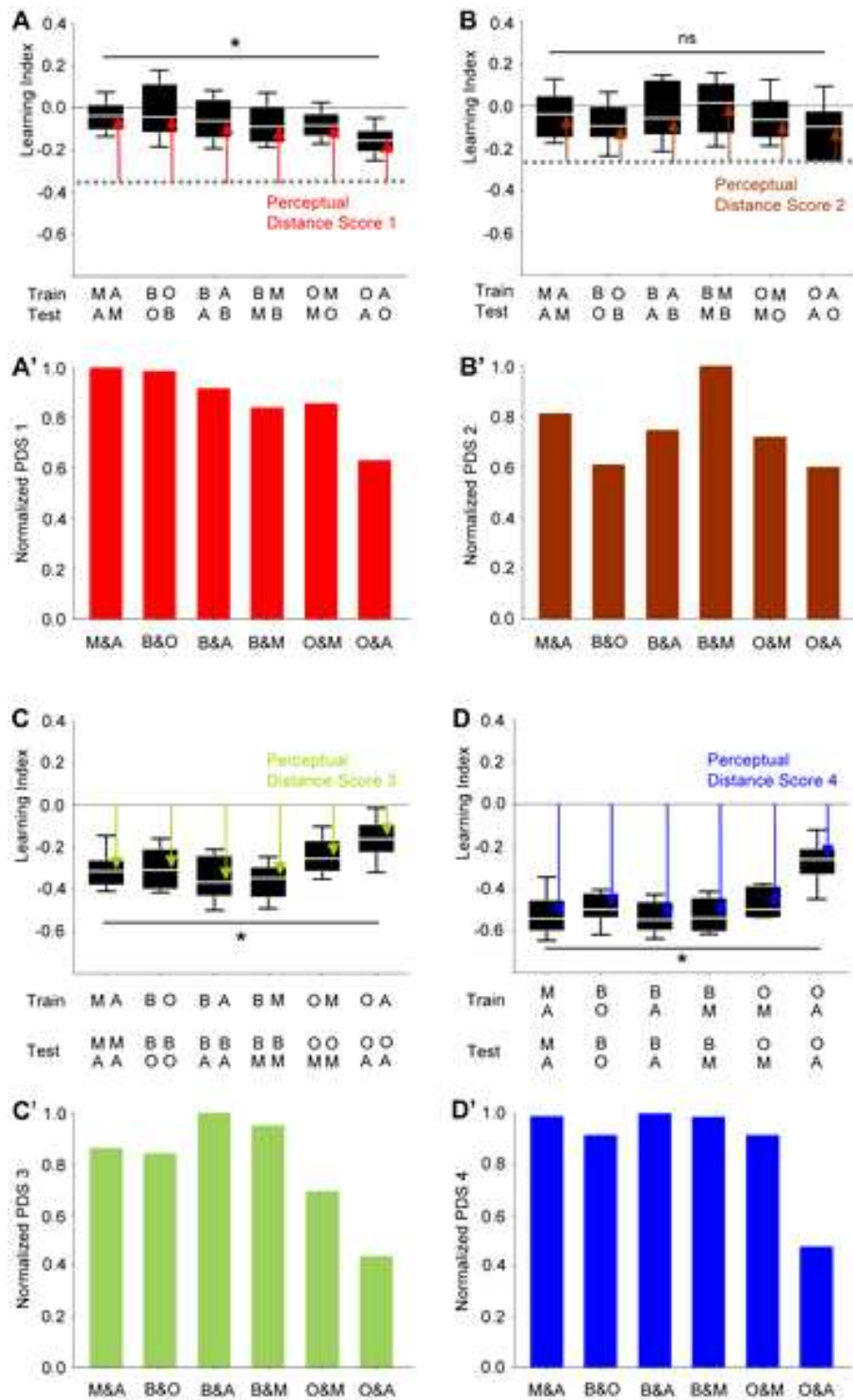
To the extent that both odours, however, were regarded as different by the flies, learning indices should increase. The level of perceived difference thus can be approximated by the Preceptual Distance Score 3 (green arrows). In (C′) these scores are presented normalized to the highest median value thus obtained. Sample sizes are from left to right: 24, 24, 20, 23, 24, 24. (D) Flies were trained such that one odour was punished but the other odour was not punished; then, flies were tested for their choice between these two odours. Thus, if the flies could not tell the two odours apart, scores should be zero. To the extent that both odours, however, could be discriminated by the flies, learning indices should increase. The level of perceived difference thus could be approximated by the Preceptual Distance Score 4 (blue arrows). In (D′) these scores were presented normalized to the highest median value thus obtained. Sample sizes are from left to right: 15, 11, 12, 11, 11, 12. * and ns refer to $P<>0.05$ in Kruskal-Wallis tests. Other details as in Fig. A.1.

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Task (iv). We trained flies to discriminate between two odours, such that during training one of the two odours was presented together with electric shock, whereas the other odour was presented without shock. At test we then presented both odours in a choice situation. The more different both odours were regarded by the flies, the easier it should have been to make a difference between them. Thus, perceived distance should have translated into easy discrimination and hence high learning scores (Fig. A.3D, D′). We find that again flies regarded O and A as least distant.

We then combined the normalized perceived distance scores from all four tasks (Fig. A.3A′–D′), and derived their median to yield a task-independent perceived distance score for each odour pair (Fig. A.4A). This showed that O and A were consistently regarded as the least distant. Because the likelihood for *any one* odour pair having the smallest distance *in all four tasks* is $P = 1 \times 1/6 \times 1/6 \times 1/6 = 0.004$, we believe that independent of task, O and A reliably have the lowest perceptual distance of our odour set.

When the physico-chemical distances between odour pairs, which consider a large number of molecular properties (Haddad *et al*. 2008) were calculated, we noted that the smallest distance in these physico-chemical scores was found for O and A, too (Fig. A.4B). This prompted us to enquire into the similarity of the patterns of physiological activity evoked by these odours.
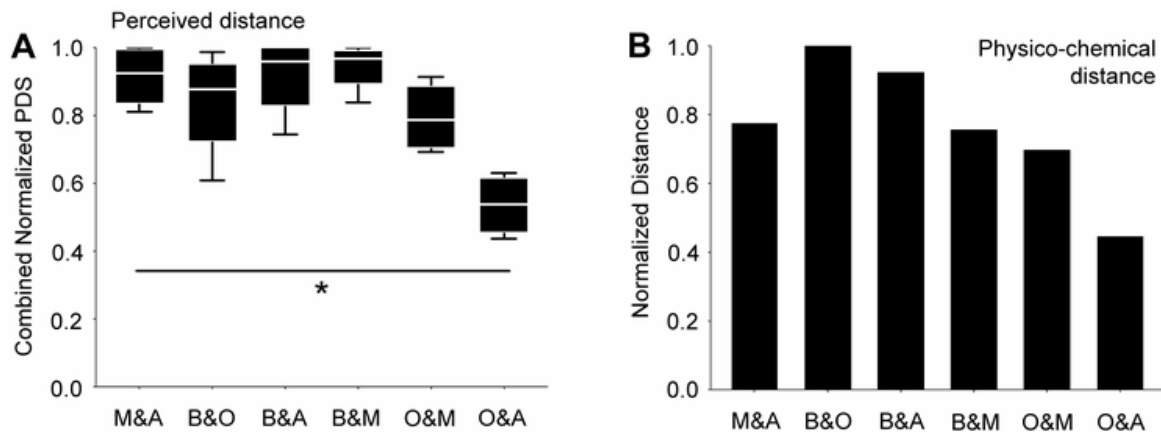

Figure A.4. Perceptual and physico-chemical distances. (**A**) The normalized Perceptual Distance Scores (from Fig. A.3A′–D′) were combined for each odour pair and presented as box plot. Note the small perceived distance between O and A. * refers to P<0.05 in a Kruskal-Wallis test; N = 4, 4, 4, 4, 4, 4. (**B**) Distances between odour pairs were derived from a physico-chemical description [23]; O and A appeared particularly similar in this kind of analysis, too. Other details as in Fig. A.1.

Physiology

The DNA-encoded fluorescence calcium sensor cameleon 2.1 (Fiala *et al*. 2002, Fiala and Spall 2003, Miyawaki *et al*. 1999) was expressed either in large populations of first- or in second-order olfactory neurons, *i.e.* either in sensory neurons or in projection neurons. Odour-evoked increases in calcium levels in these respective populations of cells were measured at the antennal lobes, the site where the sensory neurons relay onto the projection neurons. To avoid potential intensity artefacts we used the same odorant dilutions as for the behavioural experiments. Each individual fly was presented with all four odours. On the one hand, this enabled us to determine, for each animal and odour pair, the distances between the evoked activity patters (see below). On the other hand, the requirement to probe each fly with all odours limited the total number of odours that could reasonably be included in such an analysis.
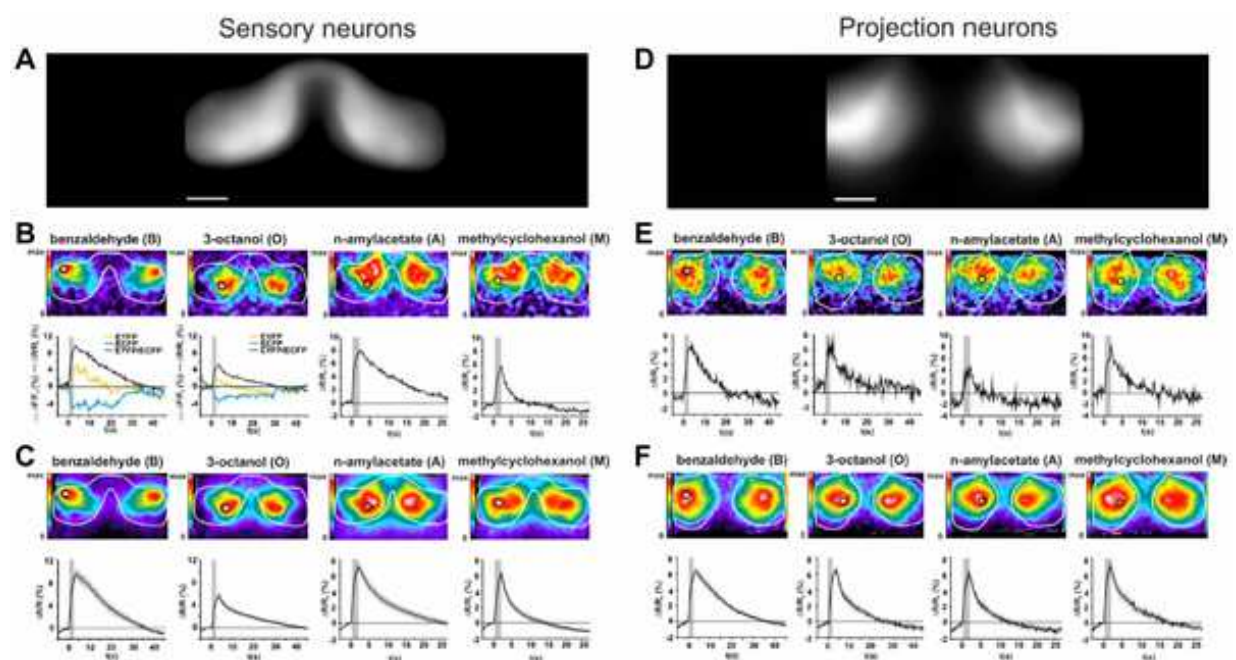

Figure A.5. High signal quality and low inter-individual variability in physiology. (**A**) To illustrate the shape of the antennal lobe as apparent in measurements of the sensory neurons, EYFP emission averaged across 8 individual flies is presented. Scale bar 25 µm. (**B**) Calcium activity recorded in the antennal lobes (white circumference-line) in sensory neurons of an individual fly after a single stimulation with the indicated odours, displayed in false-colour (top). For a defined region of interest (black circle), the time course of the measurements is displayed (bottom) as the EYFP/ECFP ratio (black). For benzaldehyde and 3-octanol as examples, also the EYFP (yellow) and ECFP (cyan) signals are plotted. The grey bar indicates the duration of the odour stimulus. (**C**) Calcium activity in olfactory sensory neurons averaged across 3–5 stimulations for each odour and in 8 individual animals displayed in false-colour (top). For the region of interest (black circle), the time course of calcium activity is displayed for the ration EYFP/ECFP (bottom). The grey bar indicates the duration of the odour stimulus. Data represent mean ± SEM. (**D, E, F**) Same as **A**, **B**, **C**, but for antennal lobe-measurements of projection neuron activity.

Regarding olfactory sensory neurons, Figure A.5 shows that calcium signals in the antennal lobe were odour-specific, spatially restricted, bilaterally symmetric, and showed remarkably high signal-to-noise ratio. Glomerular structures, however, cannot be reliably resolved with the employed technique, preventing the identification of the activated glomeruli. However, the odour-evoked patterns of activity were stimulus-specific and consistent across individuals, allowing us to compare the activity patterns, averaged across individual flies, between the four odours. Obviously, the four odours evoked distinct activity patterns at the input stage to the antennal lobe (Fig. A.6A), with the activation by O nested within the pattern evoked by A. In order to subject these activity patterns to quantitative analysis, we performed a pixel-wise principal component analysis (PCA), graphically represented by the first three principal components, covering more than 90% of the variability in the dataset (Fig. A.S3). In such a PCA, data from the eight experimental flies clustered separately for each of the four odorants (Fig. A.6A′). Notably, this PCA did not uncover a particularly low distance between O and A on the sensory neuron level (Fig. A.6A″).

False-colour coded calcium activity patterns in the antennal lobes (**A, B**), the respective PCAs (**A′, B′**), and Euclidian PCA distances (**A″, B″**) evoked by four different odorants in sensory neurons (**A- A″**) or projection neurons (**B- B″**). (**A, B**) Images present averages of eight individual flies, and 3–5 stimulations with the respective odour. Data are normalized to the maximum signal of the averaged image. The white lines indicate the outline of the antennal lobes as labelled by the respective Gal4-line (*Dmel/Orco*-Gal4, formerly known as *Or83b*-Gal4,or *GH146*-Gal4, respectively). Note that in the sensory neurons, the activity pattern evoked by O is nested within the one evoked by A; however, in the projection neurons O and A evoke the same pattern of activity. Please note that **A** and **B** re-present the data from Fig. A.5 C and F, respectively. (**A′, B′**) Pixel-wise principal component analyses across odour-evoked calcium activity within the antennal lobes as measured from sensory neurons (**A′**) or projection neurons (**B′**). Different colours indicate different odorants as indicated. Each coloured circle indicates a measurement of an individual animal. Note that in projection neurons, but not in sensory neurons, the activity patterns evoked by O and A coincided. (**A″, B″**) Euclidian distances on the basis of the first three principal components for each pair of odours were determined for each fly; these distances were combined across flies, and displayed normalized to the highest median distance thus obtained. O and A did not appear particularly similar in sensory neurons (**A″**), but turned out as the least distant odour pair in projection neurons (**B″**). * refers to P<0.05 in Kruskal-Wallis tests probing for differences across all odour pairs; N = 8 in all cases. Other details as in Fig. A.1.
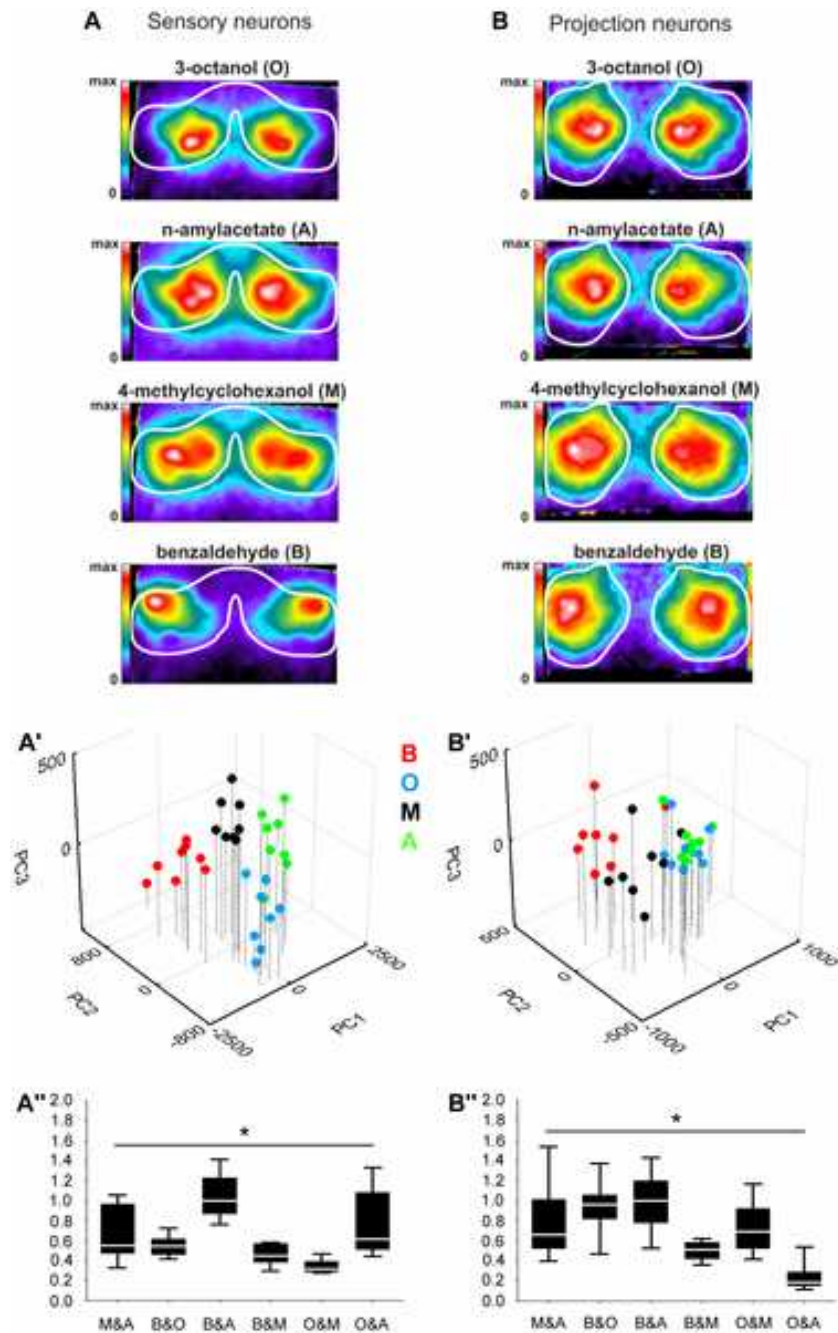
Figure A.6. Quantitative analysis of activity patterns in first- and second-order olfactory neurons. False-colour coded calcium activity patterns in the antennal lobes (**A, B**), the respective PCAs (**A′, B′**), and Euclidian PCA distances (**A″, B″**) evoked by four different odorants in sensory neurons (**A- A″**) or projection neurons (**B- B″**). (**A, B**) Images present averages of eight individual flies, and 3–5 stimulations with the respective odour. Data are normalized to the maximum signal of the averaged image. The white lines indicate the outline of the antennal lobes as labelled by the respective Gal4-line (*Dmel/Orco*-Gal4, formerly known as *Or83b*-Gal4,or *GH146*-Gal4, respectively). Note that in the sensory neurons, the activity pattern evoked by O is nested within the one evoked by A; however, in the projection neurons O and A evoke the same pattern of activity. Please note that **A** and **B** re-present the data from Fig. A.5 C and F, respectively. (**A′, B′**) Pixel-wise principal component analyses across odour-evoked calcium activity within the antennal lobes as measured from sensory neurons (**A′**) or projection neurons (**B′**). Different colours indicate different odorants as indicated. Each coloured circle indicates a measurement of an individual animal. Note that in projection neurons, but not in sensory neurons, the activity patterns evoked by O and A coincided. (**A″, B″**) Euclidian distances on the basis of the first three principal components for each pair of odours were determined for each fly; these distances were combined across flies, and displayed normalized to the highest median distance thus obtained. O and A did not appear particularly similar in sensory neurons (**A″**), but turned out as the least distant odour pair in projection neurons (**B″**). * refers to $P<0.05$ in Kruskal-Wallis tests probing for differences across all odour pairs; $N = 8$ in all cases. Other details as in Fig. A.1.

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What about the projection neurons? Odour-evoked activity patterns for O, M, and B were more widely distributed across the antennal lobe when compared to the sensory neurons (*e.g.* Fig. A.5B *versus* Fig. A.5E) and appeared less consistent between individual flies (see below). Activity patterns, however, still were sufficiently local and conserved across individual flies to allow averaging across animals and comparing these averaged activity patterns between odours (Fig. A.6B). A PCA confirmed that data of individual odours were distributed relatively more widely than is the case for the sensory neurons, reflecting the above-mentioned higher inter-individual variability (Fig. A.6B′) and presumably also the more widely distributed arborisations of projection neurons in the antennal lobe. Importantly, in this projection-neuron based PCA approach, the data for O and A formed one merged cluster (Fig. A.6B″).
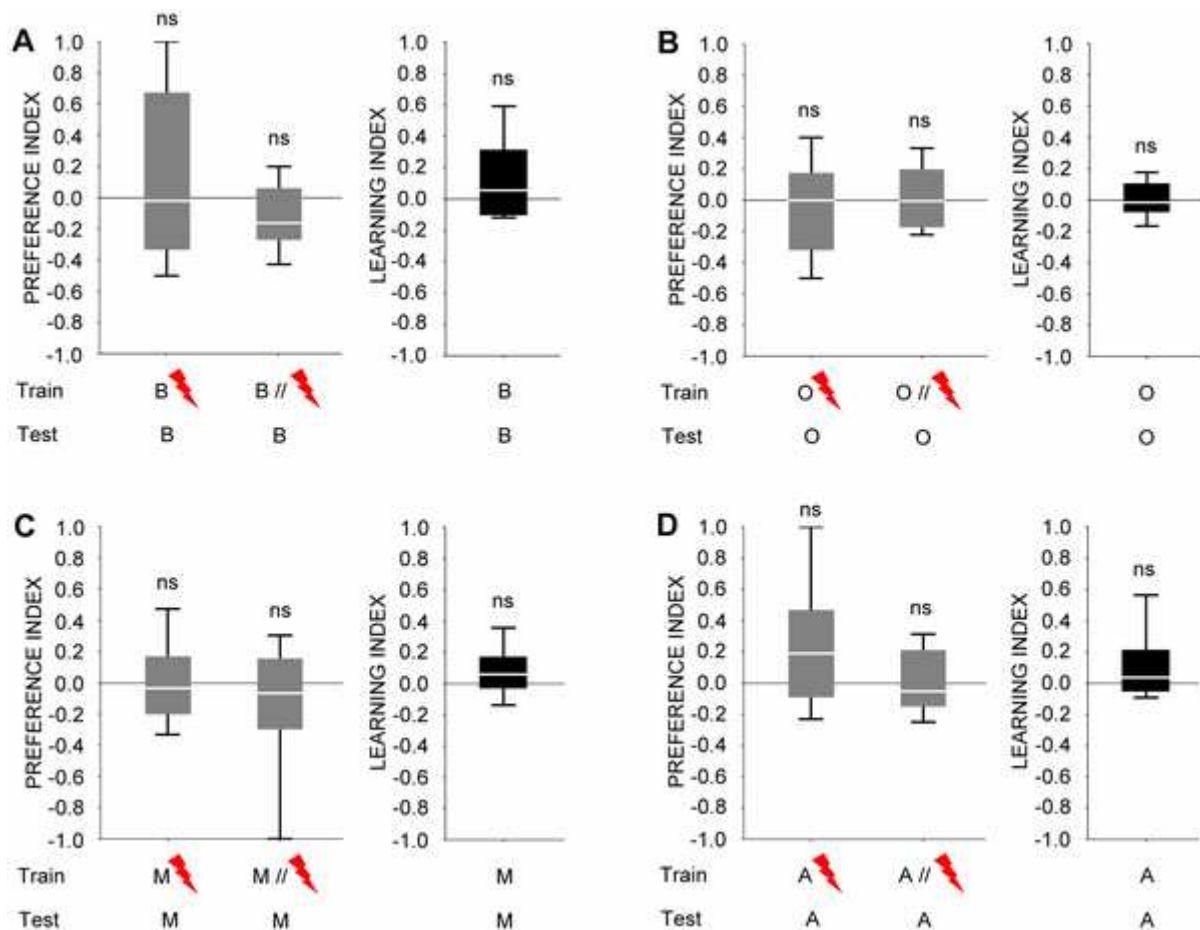

Figure A.7. A *Dmel/Orco* loss-of-function mutant (formerly known as *Or83b²*) is anosmic for all odours used. (**A**), (**B**), (**C**), and (**D**) show preference indices (grey fill) for respectively benzaldehyde, 3-octanol, 4-methylcyclohexanol, and *n*-amylacetate after odour-shock training, and the corresponding learning indices (black fill). Neither preference indices nor learning indices are different from zero in the *Dmel/Orco* mutant, suggesting an absolute requirement of *Dmel/Orco* for processing of these odours. Thus, a lack of correspondence between perception and sensory-neuron physiology cannot be attributed to processing outside of the *Dmel/Orco*-Gal4 expression pattern.

Thus, the low perceptual distance for O and A (Fig. A.4A) did not apparently conform to sensory-neuron distances (Fig. A.6A″) (this lack of match was not due to processing outside the sensory neuron driver (*Dmel/Orco*-Gal4, the driver formerly known as *Or83b*-Gal4, because *Dmel/Orco* loss-of-function mutants were anosmic for all odours used: Fig. A.7). However, in the projection neurons a low distance between O and A was revealed (Fig. A.6B″). Therefore, the processing step from first- to second-order olfactory neurons apparently corresponds to a categorization step, making the activity patterns for O and A more similar. In our dataset, this came about by a sharpening of the activity pattern evoked by A such that, while at the level of the sensory neurons the signal evoked by O was nested within the one evoked by A, both odours activated almost fully overlapping areas of the antennal lobe when the projection neurons were considered (Fig. A.6).

Discussion

The relationship between olfactory perception and physiology has been elegantly studied in the honeybee (Guerrieri *et al*. 2005): One out of 16 odours was trained by presenting it together with a sugar reward, and, for any individual bee, testing for conditioned proboscis extension was carried out with a random draw of four from these 16 odours to generate a 16-dimensional behavioural odour space. Euclidian distances between odour pairs could thus be used for a correlation analysis with similarities of physiological activity patterns in the antennal lobe as had been measured earlier using bath-applied calcium dyes (Sachse *et al*. 1999). In agreement with what we report here, behavioural and physiological distances between odour pairs matched fairly well. However, using bath-applied dyes does not allow one to assign cellular identity of the measured cells with reasonable certainty. Also, behavioural scores were in a number of cases asymmetrical: Response levels to aldehydes were generally high after training to odours of other functional classes (primary and secondary alcohols, ketones), whereas after training with aldehydes response levels to odours from these other classes were low. Such asymmetries can result from not adjusting odour intensities for equal learnability (and/or from the repeated testing of individual bees). For example, suppose for task (i) an odour X would have high learnability, whereas odour Y would be less learnable at the respective dilution used. One may then find strong conditioned avoidance of Y after training with X because the memory for X is strong and because X and Y are regarded as similar to a particular extent. However, after training with Y, conditioned avoidance of X may be low simply because the memory for Y is weak and although X and Y actually are regarded as similar. This would entail an apparent asymmetry of similarity

judgments, violating a fundamental property of metrics in a mathematical sense (the distance between X and Y cannot be different from the distance between Y and X).

Our findings may at first sight appear inconsistent with the report of Kreher *et al.* (2008). The authors measured odour-induced electrophysiological activity in adult *Drosophila* olfactory sensory neurons which express, rather than their cognate *Or* gene, only one of the 21 larval-expressed *Or* genes. This was done for all these 21 larval *Or* genes and a panel of 26 odours to obtain a physiological odour space. Behaviourally, the authors assayed larval *Drosophila* in a masking experiment: One odour was presented as a point source within a background mask of another odour present throughout the experimental arena. If a larva responds to the point source despite the mask, it must have the ability to tell apart the point source from the mask. Regarding odour quality processing, the argument requires that no behavioural responses to the point source are seen if the same odour is used as both point source and mask. This was shown to be the case for four out of the six odours thus assayed. Notably, results were in some cases asymmetrical (*e.g.* ethyl butyrate and 2,3-butanedione; see discussion above). Still, perceptual similarity thus measured correlates with the distances between odours in the physiological odour space. This is not a contradiction to our findings, however, because focussing on the sensory neurons may not reveal potentially better matches between physiology and perception in the projection neurons. Also, different sites along the olfactory pathway may be important for different kinds of behavioural similarity judgements: Masking may come about on the level of sensory neurons and thus the physiology of these very neurons may underlie masking-based measures of perception, while more central processing stages may be involved in recognition-type measures of perceived similarity, as in our case.

We note that the distances of odour pairs in perception (Fig. A.4A), in terms of physico-chemical distance (Fig. A.4B), and projection-neuron physiology (Fig. A.6B″) all suggest O and A to be relatively similar. This may imply that the actual physico-chemical parameters of odours are not as such given in sensory neurons, but need to be derived as processing progresses. In the case of O and A, this apparently entails a classification of sensory inputs according to their overall physico-chemical similarity. Perception and ensuing behaviour seem to be based on these processed, second-order categories. Admittedly, the correspondences between perceptual distance, projection-neuron physiological distance, and physico-chemical distance are coarse (see for example the odour pair B and M), within this as

well as earlier (Kreher *et al*. 2008, Guerrieri *et al*. 2005) studies. This may be due to differences in genotype between behavioural and physiological measurements, imperfections and/or incompleteness of physiological measurements, the kind and number of odours sampled, and/or due to specific demands imposed by the respective behavioural assays. Also, processing stages downstream of sensory and projection neurons, such as the mushroom bodies, and/or temporal aspects of physiological activity likely contribute to shape perception. These caveats in mind, finding even a coarse match between perception, physiology at any one processing step, and physico-chemical odour features is actually surprising. The employed widefield microscopy to determine calcium activity patterns in *both* antennal lobes makes it difficult to identify the activated glomeruli because calcium signals are detected from different depths of the preparation. Therefore, we intentionally refrain from referring calcium activity patterns to identified glomeruli. Rather, we apply a more unbiased method and describe the similarity between odour-evoked calcium activity patterns on the basis of pixels. In the future, it will be of interest to use high-resolution microscopy (*e.g.* 2-photon-imaging) to determine in detail the anatomical substrates as well as the underlying circuit architecture which causes a catergorization of odour stimuli.

Thus, based on our results we suggest that within-antennal lobe processing may organize odour-evoked activity according to the physico-chemical properties of the odours, and that this process may be a basis for the flies' behavioural similarity judgements. Regarding these judgements it seems important to note that along the olfactory sensory-motor loop olfactory signals, gradual in nature, eventually have to be dichotomized by the flies in order to 'decide' whether to run away from a given odour- or not. The first steps in this process to funnel olfactory representations into behavioural categories, we suggest, may already be taken at the level of the antennal lobe, according to the physico-chemical properties of the odours. Given that so far the antennal lobe network has mostly been implicated in maintaining or even enhancing distinctiveness between odours [discussion in 13], such a categorization process would provide a novel aspect of antennal lobe function.

Materials and Methods

Behaviour

Wild-type Canton-S flies were kept in mass culture at 25°C, 60–70% humidity and a 14/10 hour light/dark cycle. For the data displayed in Fig. A.7, an *Dmel/Orco* loss-of-function

mutant (Larsson *et al*. 2004) (the mutant formerly known as *Or83b²*) was used (Bloomington stock center, #23130). Flies were collected one to five days after pupal hatching and kept over-night at 18°C.

Training was performed in dim red light, testing in darkness. As stimuli we used benzaldehyde, 3-octanol, 4-methylcyclohexanol, or *n*-amylacetate (B, O, M, A) (CAS: 100-52-7, 589-98-0, 589-91-3, 628-63-7; all from Fluka, Steinheim, Germany, except A, which is from Merck, Darmstadt, Germany), or ambient air (Θ). This odour choice was based on the *Drosophila* learning literature since the 70 s; we thus probably sampled a subset of relatively easily discriminable odour pairs. A vacuum pump ensured removal of odour-saturated air from the training apparatus. Odorants (130 µl) were applied in Teflon cups of 7-mm diameter either in pure condition or diluted in paraffin oil (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000, unless mentioned otherwise) (paraffin oil from Merck). At t = 0 min, groups of about 100 flies were loaded to the training tubes of the experimental apparatus which allowed applying electric shock via an electrifiable grid covering the tube. At t = 2 min, the first stimulus (either B, O, M, A, orΘ) was presented for 60 s without punishment. At t = 4 min, the second stimulus (any of the remaining four) was presented for 60 s; 15 s after stimulus onset, an electric shock was applied (90 volts, 12 pulses á 1.2 s within 60 s, onset-onset interval 5 s). At t = 9:00 min, flies were transferred back to their food vials for 13 min until the next of the in total three such training cycles starts. Across independent measurements, the sequence of events was either as indicated during all three training cycles, or was reversed such that the first stimulus presented was punished.

After training, the regular 13 min break was given (unless mentioned otherwise). After an accommodation period of 4 min, animals were transferred to an appr. 1.5 cm³ choice chamber of a T-maze, from where they could escape towards either of two of the five above-mentioned stimuli. After 2 min, the arms of the maze were closed, the number of animals within each arm (denoted #) counted, and the relative preference between the choice options determined as documented in Fig.s S5, S6, S7, S8, S9. A preference index (PI) was calculated as:

$$PI = (\#_{\text{Punished stimulus}} - \#_{\text{Non-punished stimulus}}) / \#_{\text{Total}} \qquad (1)$$

A second set of flies was trained reciprocally: If *e.g.* in Experiment (iv) (Fig. A.3D), one set of flies was punished when receiving M but not when receiving A, the second set of

flies was trained by presenting A with but M without punishment. The same reciprocity was followed in all tasks. PIs of these two reciprocally trained sets of flies were then averaged to obtain a learning index (LI). Thus, positive LIs indicate conditioned approach, negative LIs conditioned avoidance. Data are presented as box plots with the middle line showing the median and box boundaries and whiskers the 25%/75% and 10%/90% quantiles, respectively, and were analysed with non-parametric statistics (Statistica, Statsoft, Hamburg, Germany), using a Bonferroni correction as applicable. Flies were trained and tested only once.

After adjusting odour dilutions for equal learnability (Fig. A.1; Fig. A.2A), four tasks were performed:

1. In a 4×4×2 experimental design, flies were trained with any one of the four odours *versus* Θ. Then, they were tested either for their avoidance of the trained odour, or of any one of the remaining three non-trained odours, *versus* Θ. This was done either after the regular 13-min break (i), or after an additional 180-min waiting period (ii).

2. Flies were trained as in (i), but were tested in a two-odour choice situation for their relative preference between the punished *versus* any of the three non-punished odours.

3. Flies were trained differentially between two odours and were then tested for their relative preference between them in a two-odour choice situation.

Physico-chemical distances

We used the odour metric as presented by Haddad *et al*. (2008). Odour structures were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and input to the Dragon software (http://www.talete.mi.it/products/dragon_description.htm). In the used version 5.4, this metric represented each odorant as vector of 1664 molecular descriptor values and yielded, for the respective odour pairs, the following values: M-A: 28.6755; B-O: 37.0393; B-A: 34.1564; B-M: 27.9832; O-M: 25.8083; O-A: 16.5091. In Fig. A.4B, these scores are presented normalized to the highest value thus obtained. We note that when using a second, independent metric (Schmuker and Schneider 2007, Schmuker *et al*. 2007), the pattern of results was the same (not shown; pers. comm. Michael Schmuker, Freie Universität Berlin).

Physiology: Optical calcium imaging

Cameleon 2.1 (Miyawaki *et al*. 1999) was expressed using either *Dmel/Orco*-Gal4 (formerly known as *Or83b*-Gal4) (Larsson *et al*. 2004), or *GH146*-Gal4 (Stocker *et al*. 1997).

All animals were homozygous for both the UAS:*cameleon* insertion (Diegelmann *et al*. 2002: strain 82) and the respective Gal4 insertion.

5–7 day-old female flies were briefly cooled on ice for immobilization and restrained by inserting them into a truncated pipette tip with the head sticking out. The fly was glued with its head under a transparency foil and then fixed on a plastic cover slip using dental glue (Protemp II, 3M ESPE, Seefeld, Germany). The third antennal segments and maxillary palps remained dry and untouched. A window was cut into the head capsule and the hole covered by a drop of Ringer's solution (Estes *et al*. 1996). The preparation was placed under an upright widefield fluorescence microscope (Zeiss Axioscope 2 FS) equipped with a 40× water immersion objective (Zeiss Achroplan) (Zeiss, Göttingen, Germany) and a cooled CCD camera (CoolSnap HQ, Photometrics, Pleasanton, CA). Excitation light of 436 nm was provided by a xenon lamp and a grid monochromator (Visitron Systems, Puchheim, Germany). Fluorescence emission was guided through a 455 nm DCLP pass filter (Chroma Technologies, Rockingham, VT, USA); the wavelengths of EYFP and ECFP emission (530 nm and 480 nm, respectively) were separated using a beam splitter (Optical Insights, Santa Fe, NM, USA) equipped with a cameleon filter set (Chroma Technologies, Rockingham, VT, USA). The two half-images of EYFP and ECFP emissions were simultaneously recorded by the two halves of the CCD chip (1392×1040 pixels) at a binning of 4×4, resulting in one stored image of 174×260 pixels per time frame and wavelength. After binning, each stored pixel was a 14-bit real number reflecting the image intensity of the respective wavelength. Images were acquired at a frame rate of 5 Hz (200 ms) with an exposure time of 100 ms per frame, controlled by MetaFluor software (Visitron Systems, Puchheim, Germany). Each EYFP image at time point t was labelled $S^Y(t)$, and each ECFP image $S^C(t)$, respectively.

Odour delivery was achieved using a custom-built olfactometer. A constant air stream generated by a vacuum pump was directed via a glass pipette to the fly's antennae and maxillary palps. The airstream was shunted to vials that are either blank, contained paraffin oil as solvent-control or one of the four odorants diluted in paraffin oil as for the above behavioural experiments. All flies received cycles of six stimulations each, in the order blank air, solvent, O, A, B, and M. Specifically, 2-s stimuli are applied 3 s after the onset of the experiment, followed by a 60 s break after which another stimulus was applied until the set of stimulations was complete. This cycle was repeated 3–5 times for each fly.

Quantitative data analysis

Image alignment was performed using a modified version of the ImageJ plugin TurboReg (Thevenaz *et al*. 1998) that allowed for the alignment of images without changing the value of any pixel. First, images were cropped by 5 pixels in one direction to remove a black edge produced by the beam splitter device, resulting in 169×260 pixels per image. Data analysis then was performed using a custom-written Java script implemented in ImageJ. Aligned EYFP and ECFP images were used to generate EYFP/ECFP ratio images $S(t) = S^Y(t)/S^C(t)$; all subsequent image analysis was based on this ratio signal. For calculating odour-evoked calcium signals, five frames preceding odour onset (frame 8–12; odour onset at frame 16) were averaged (prestimulus), and five frames beginning 400 ms after odour onset (frames 18–22) were averaged (stimulus). The averaged prestimulus image then was subtracted from the averaged stimulus image to obtain a calcium signal image. To reduce noise, images were filtered by replacing each pixel intensity by the average of the surrounding 8×8-pixel area. To reduce noise, the calcium signal images obtained by the 3–5 stimulations per odour were averaged for each fly measured.

Time courses of calcium signals averaged over distinct regions of interest (defined in the figures) were calculated based on the original images $S^Y(t)$ and $S^C(t)$ using the MetaMorph software (Visitron Systems, Puchheim, Germany). For time-resolved estimates of calcium activity (*e.g.* bottom of Fig. A.5B, E), fluorescent emission of EYFP and ECFP averaged over a distinct region outside the labelled structure (the 'background' outside the white circumfence line of *e.g.* top of Fig. A.5B, marked in the respective figures) was at each time point subtracted from the value within the chosen region of interest (F(t)-value: either $F^Y(t)$ or $F^C(t)$) (*e.g.* black circle in Fig. A.5B, top). For calculating changes in fluorescence ($\Delta F(t)$), the F(t) value at odour onset (F_0) was subtracted from the F(t) value at the respective time point t; $\Delta F(t)$ was then divided by F_0 ($\Delta F(t)/F_0$). To exploit the sensors' nature of increasing EYFP fluorescence and decreasing ECFP fluorescence upon increased calcium levels, which largely eliminates movement artefacts, the ratio of F(t)-values for EYFP and ECFP was calculated (EYFP/ECFP) (R(t)-value: $R(t) = F^Y(t)/F^C(t)$); thus, the normalized change in this ratio ($\Delta R(t)/R_0$) represented calcium activity. Maximum calcium activity was typically found in a time window 3 s after odour onset (*e.g.* bottom of Fig. A.5B); thus, the false-colour coded images (*e.g.* top of Fig. A.5B) represent calcium activity ($\Delta R(t)/R_0$) for each pixel at this time point.

For analyzing odour-evoked calcium activity patterns, the regions of interest (ROI) covering one antennal lobe in calcium images $S^Y(t)$ and $S^C(t)$ were first defined using thresholding (Fig. A.S4). Pixel intensities of background EYFP images were averaged and are normalized between 0 and 1 and were chosen as ROI pixels when intensities are greater than 0.40 or 0.65 for sensory neurons or projection neurons, respectively. The choice of threshold values depends on the contour of the ROI, reflecting the anatomical position of the investigated groups of neurons. Only the calcium signals within the ROI were used for further analysis.

We used Principle Component Analysis (PCA) to reduce the high-dimensional data to three dimensions that accounted for most of the variance. The principle components (PCs) were indexed according to their contribution to the total variance. Here, the calcium signals in the ROI (7575 data points for sensory neurons and 5890 data points for the projection neurons, respectively) were reduced to the three dominant principle components that turned out to keep >90% of the variability of the signals (see Fig. A.S3). Euclidian distances were computed for each pair of odours based on the first three PCs, combined across flies and displayed as box plots in Fig. A.6A″, B″ normalized to the highest median distance thus obtained.

Supplementary figures
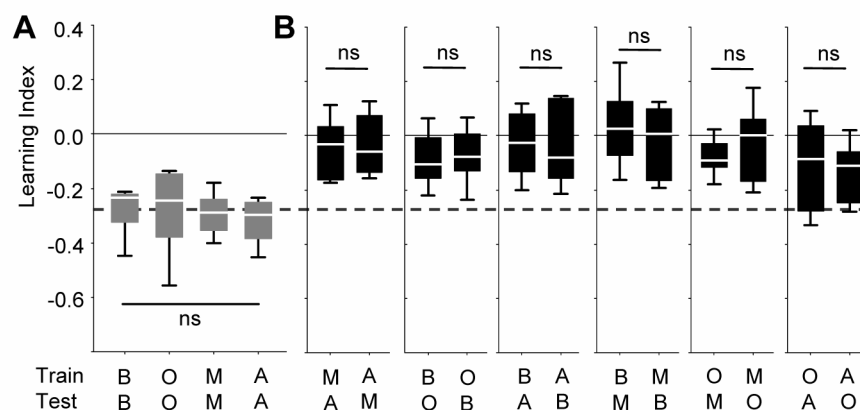
Figure A.S1. Symmetry of perceived distance measures. (A) Confirming that also after an additional retention period of 180 min learning indices are equal for the chosen dilutions of odour. Sample sizes are from left to right 8, 8, 8, 8. (B) Data from Fig. A.3B separated by odour; note that learning indices in all cases are symmetrical, in the sense that response levels *e.g.* to A after training with O are as high as response levels to O after training to A. The stippled line in (B) represents the median of the pooled data from (A) and corresponds to the one in Fig. A.3B. Sample sizes are from left to right 12, 12, 12, 12, 12, 12, 12, 12, 12, 12, 12, 12. Other details, and abbreviations of odour identity, as in Fig. A.1.
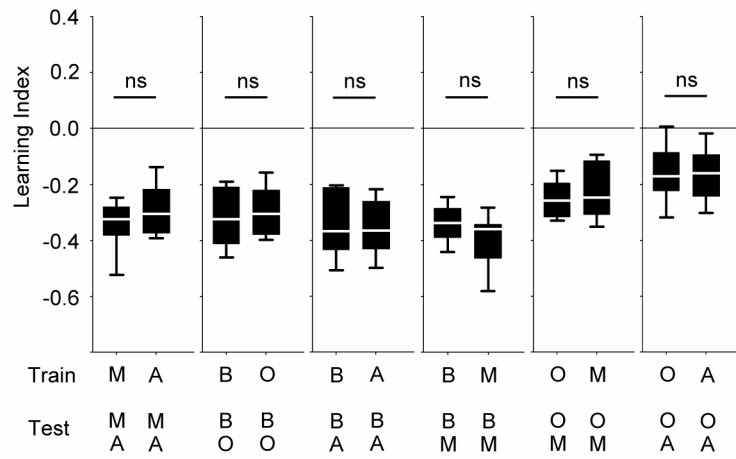
Figure A.S2. Symmetry of perceived distance measures. Data from Fig. A.3C, separated by odour. Note that learning indices in all cases are symmetrical, in the sense that learning scores are the same when choice between O and A is assayed after training to O, as they are after training to A. Sample sizes are from left to right 12, 12, 12, 12, 10, 10, 11, 12, 12, 12, 12, 12. Other details, and abbreviations of odour identity, as in Fig. A.1.
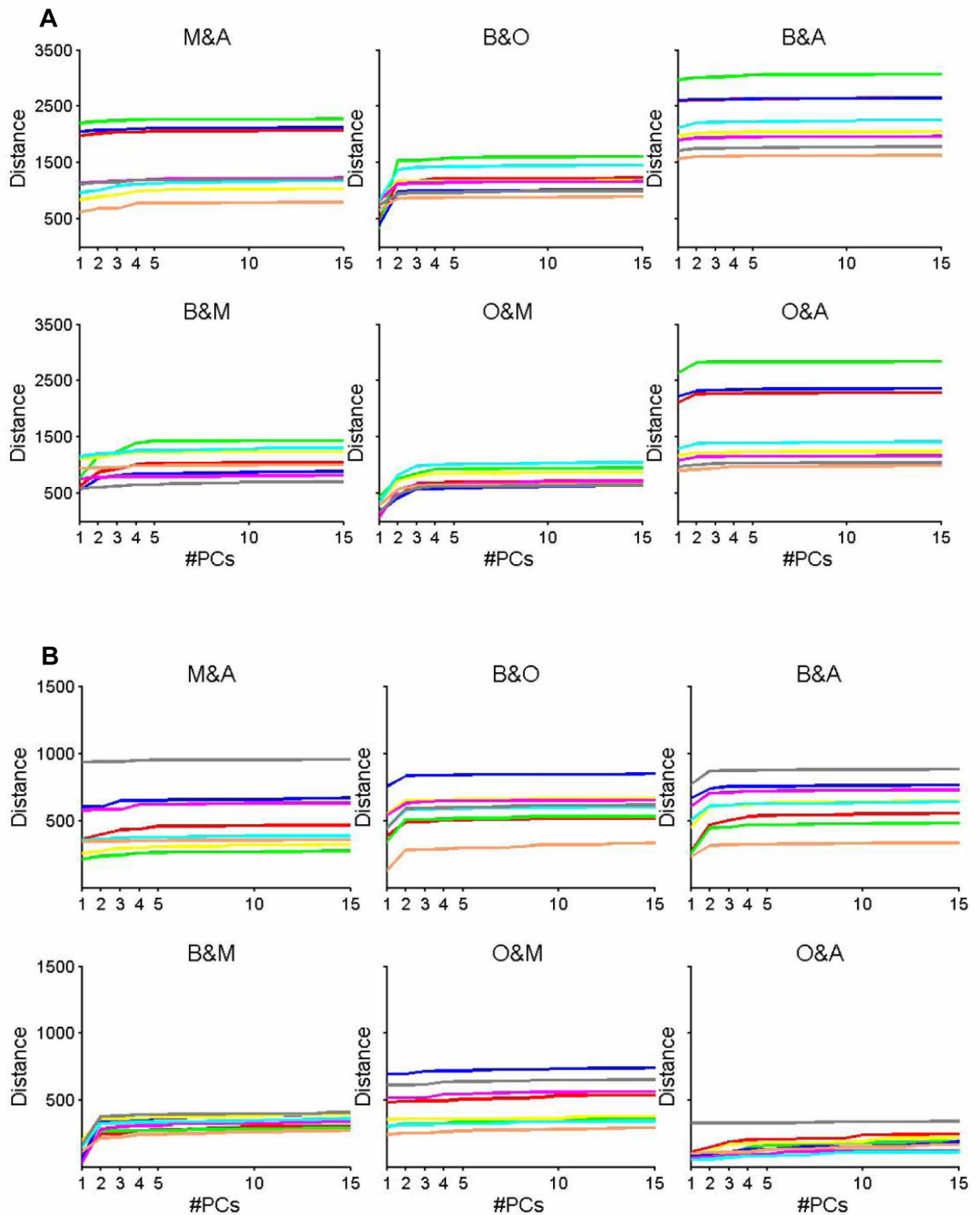
Figure A.S3. Validation of the three-PC based Euclidian distance measures. Euclidian distances of odour-evoked activity (A: sensory neurons, B: projection neurons) are computed for each pair of odours based on increasing numbers of principle components (x-axis: #PCs). The differently colored lines indicate data from individual animals. Note that for both populations of neurons the Euclidian distances remain constant or only slightly increase when using more than three principle components, demonstrating that the relative similarity between calcium activity patterns is effectively covered by the first three principle components. In other words, additional principle components do not add significant information.
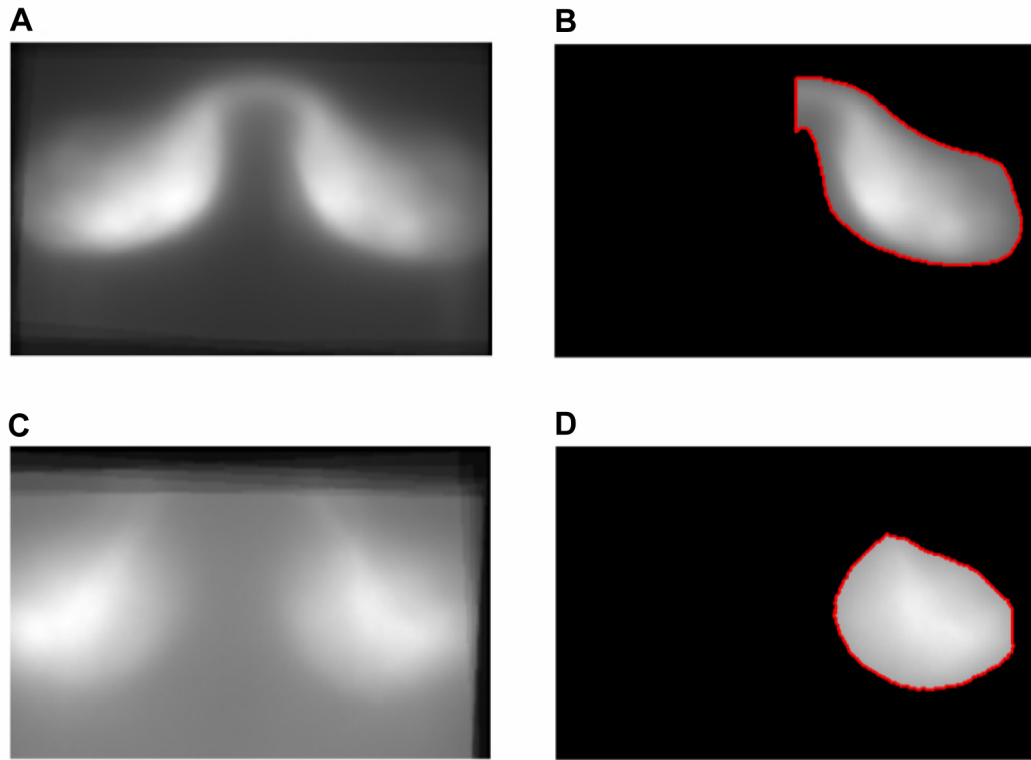
Figure A.S4. Definition of the Region of Interest (ROI) for the pixel-based PCA. (A) To define the Region of Interest (ROI) for the PCA of the sensory neurons innervating the antennal lobes across measurements, EYFP emission across 8 individual flies is averaged. (B) The region of interest used for PCA of sensory neuron activity in the antennal lobe (red circumference-line), defined by using a threshold of 0.45 of the maximum intensity value. (C) As in (A), but for the projection neurons. (D) As in (B), but for the projection neurons, except that (C, D) used a threshold of 0.60 of the maximum intensity value.
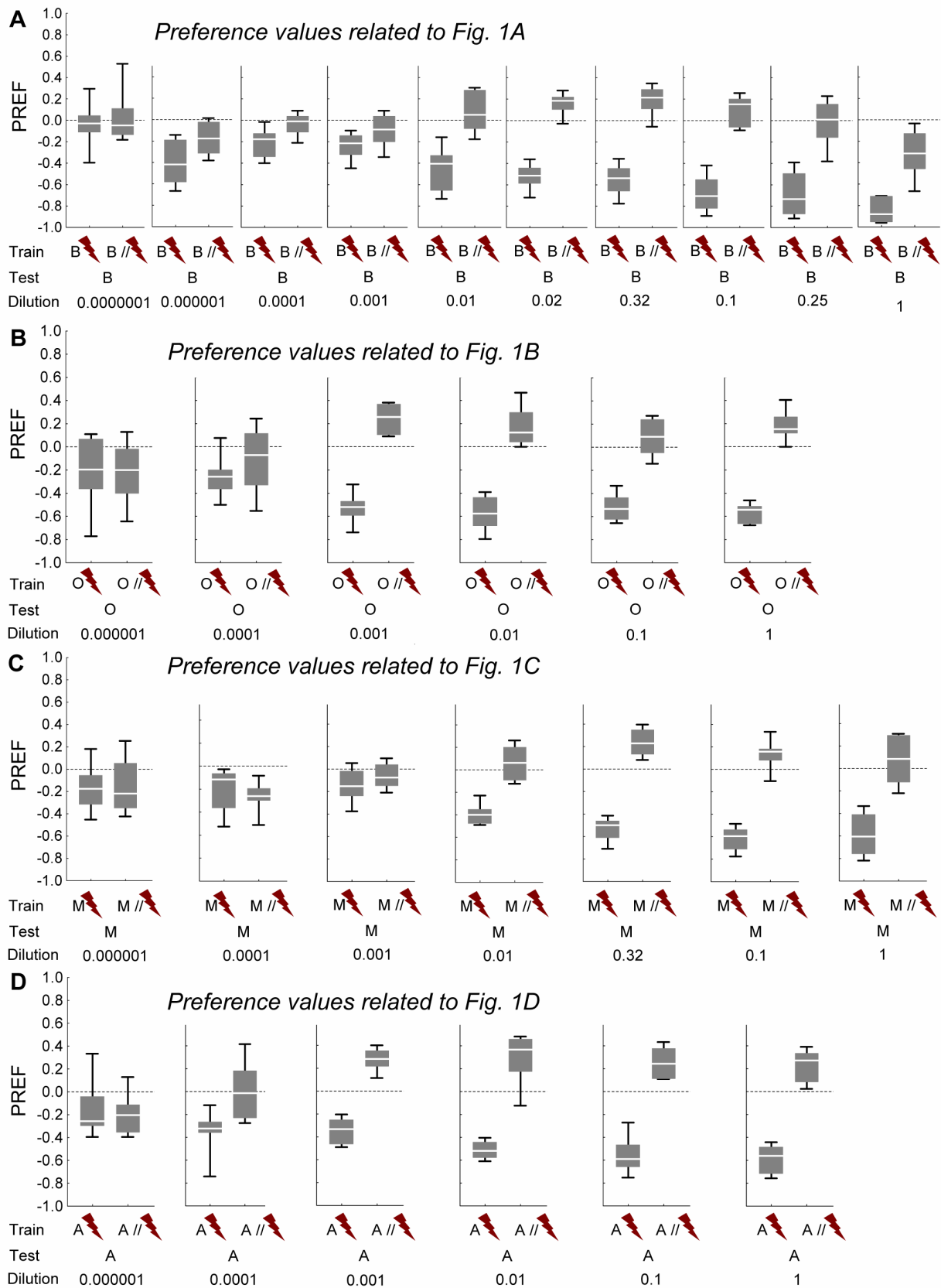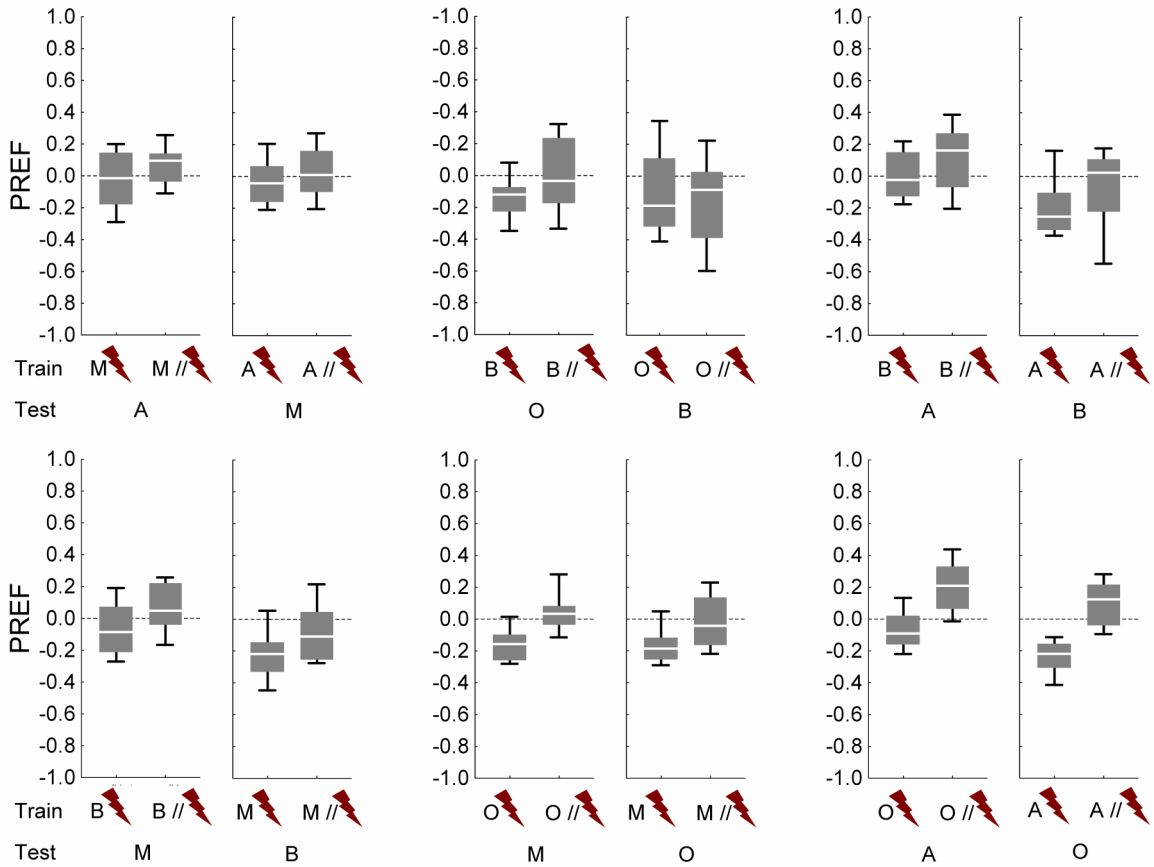
Figure A.S5. Preference scores underlying the associative performance indices shown in Figure A.1A–D. The behaviour of the reciprocally trained groups of flies as underlying the associative learning indices (LIs) of Fig. A.1A–D is documented by preferences (PREF) scores. On the basis of the the number of flies in the respective arm of the maze (#) these scores are calculated as:

$$PREF = (\#_{Odour} - \#_{No\text{-}Odour})/\#_{Total}$$

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A *Preference values related to Fig. 2A*
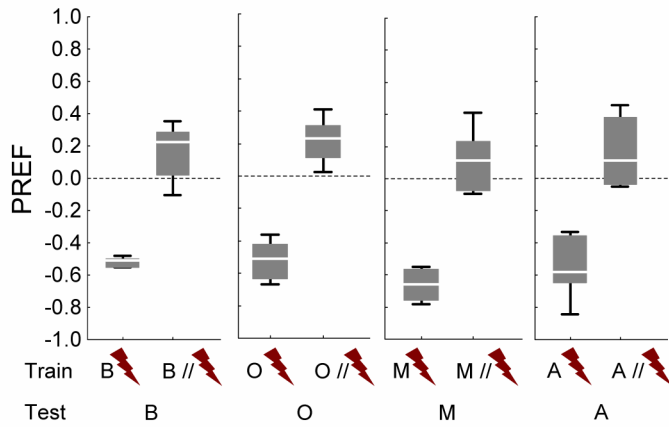
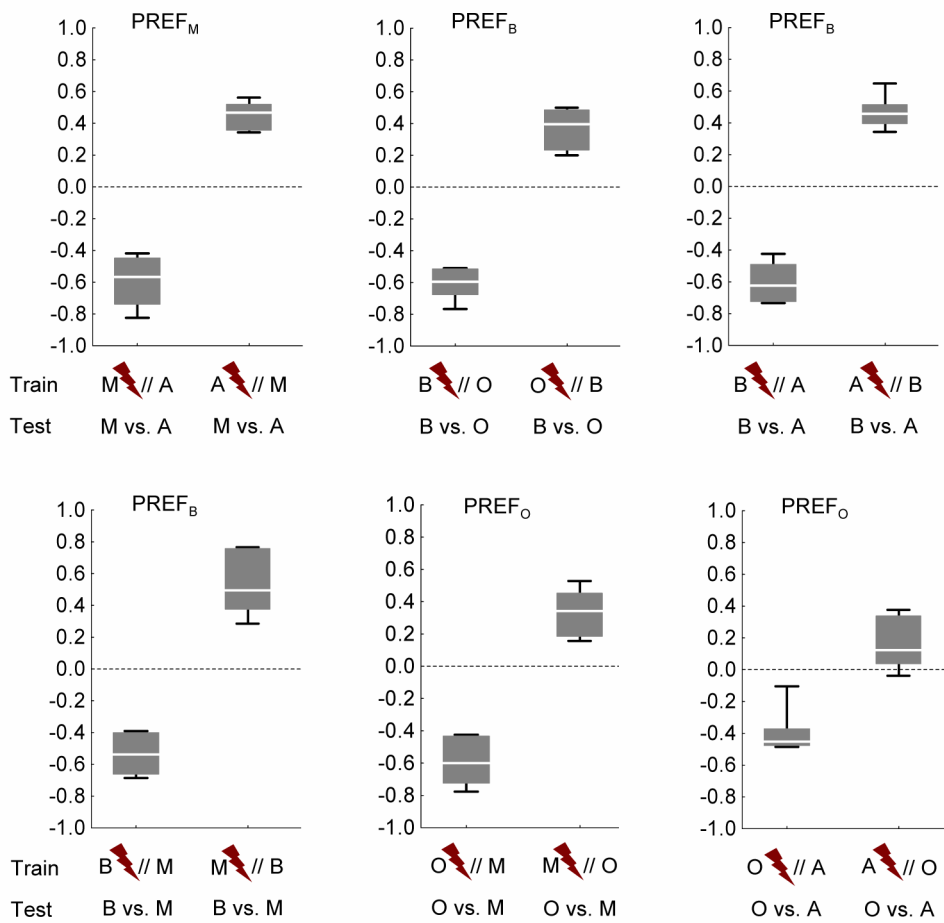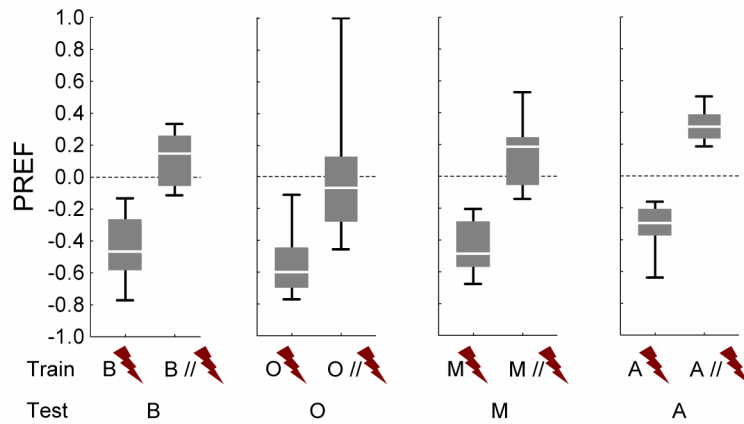**B** *Preference values related to Fig. 2B*

Figure A.S6. Preference scores underlying the associative performance indices shown in Figure A.2A–B. The behaviour of the reciprocally trained groups of flies as underlying the associative learning indices (LIs) of Fig. A.2A–B is documented by preferences (PREF) scores. On the basis of the the number of flies in the respective arm of the maze (#) these scores are calculated as:

$$PREF = (\#_{Odour} - \#_{No\text{-}Odour})/\#_{Total}$$

Figure A.S7. Preference scores underlying the associative performance indices shown in Figure A.3D. The behaviour of the reciprocally trained groups of flies as underlying the associative learning indices (LIs) of Fig. A.3D is documented by preferences (PREF) scores. On the basis of the the number of flies in the respective arm of the maze (#) these scores are calculated as:

$$PREF = (\#_{Odour\text{-}indicated\text{-}on\text{-}X\text{-}axis} - \#_{Other\ Odour})/\#_{Total}$$

A *Preference values related to Fig. S1A*
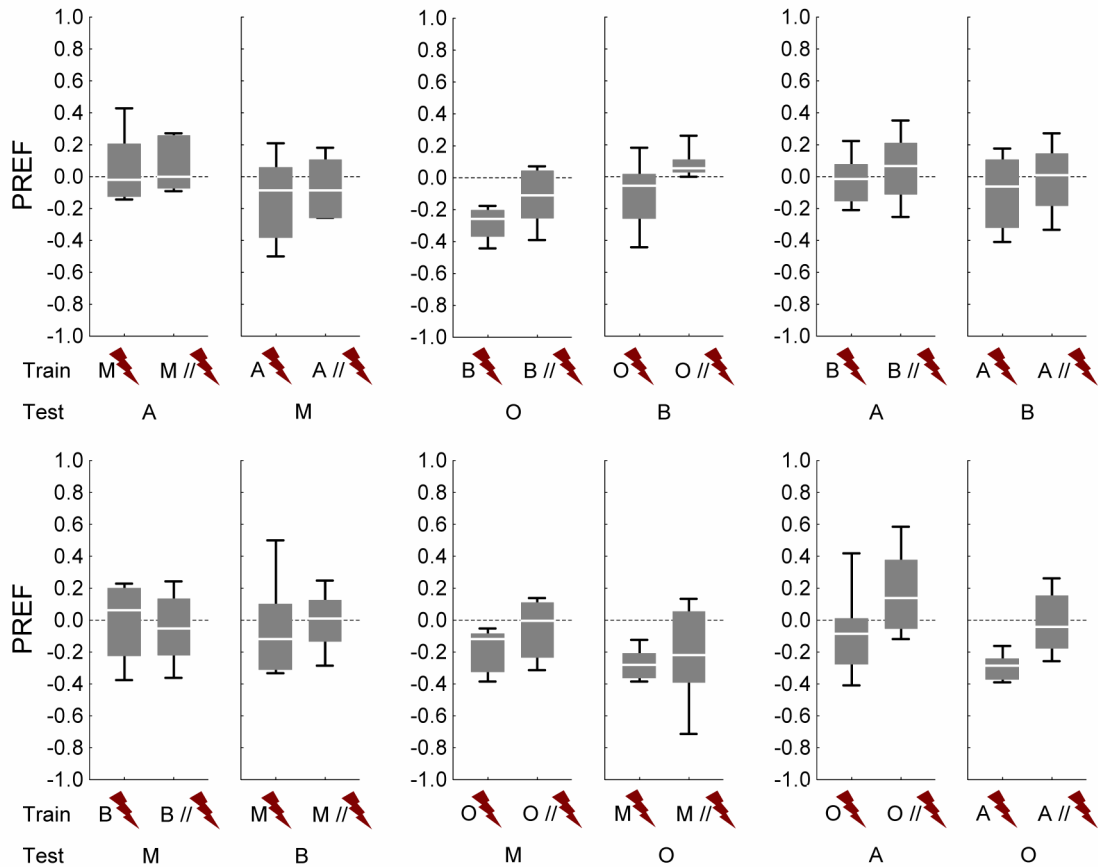

B *Preference values related to Fig. S1B*

Figure A.S8. Preference scores underlying the associative performance indices shown in Fig. A.S1A–B. The behaviour of the reciprocally trained groups of flies as underlying the associative learning indices (LIs) of Fig. A.S1A–B is documented by preferences (PREF) scores. On the basis of the the number of flies in the respective arm of the maze (#) these scores are calculated as:

$$PREF = (\#_{Odour} - \#_{No\text{-}Odour}) / \#_{Total}$$

130

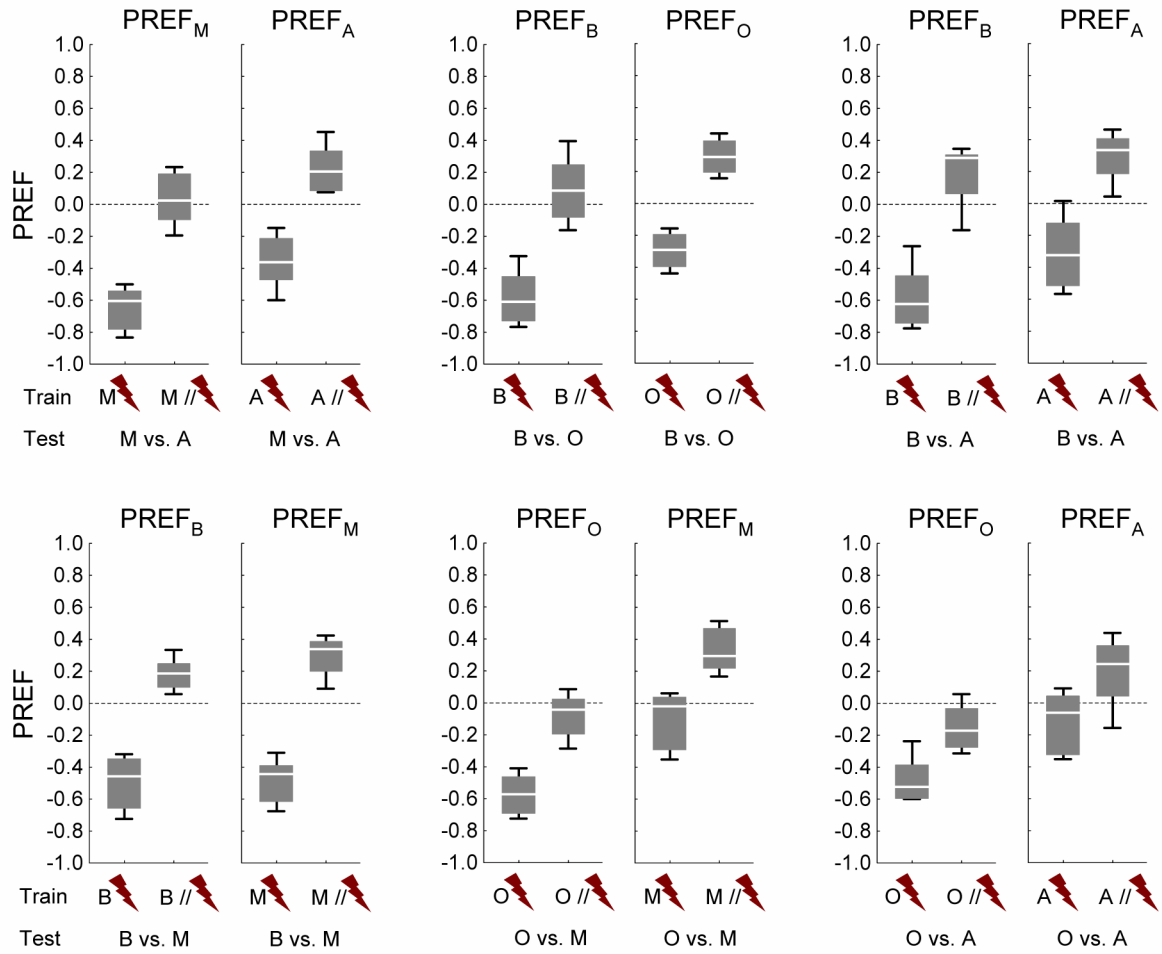*Preference values related to Fig. S2*

Figure A.S9. Preference scores underlying the associative performance indices shown in Fig. A.S2. The behaviour of the reciprocally trained groups of flies as underlying the associative learning indices (LIs) of Fig. A.S2 is documented by preferences (PREF) scores. On the basis of the the number of flies in the respective arm of the maze (#) these scores are calculated as:

$$PREF = (\#_{Odour-indicated-on-X-axis} - \#_{Other\ Odour}) / \#_{Total}$$