Integrating past, present and future:

mechanisms of a simple decision in larval *Drosophila*

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Die vorgelegte Dissertation besteht aus zwei Publikationen, zwei Publikationen „in press“ und einem zur Veröffentlichung vorbereiteten Manuskript. Eingeleitet wird sie durch eine allgemeine Einleitung und Diskussion und abgeschlossen durch einen Outlook. Die Mitwirkung der Co-Autoren jeder Publikation wird auf den folgenden Seiten herausgearbeitet. Ich versichere, dass alle Co-Autoren mir die Einwilligung erteilt haben, die entsprechenden Mansukripte in dieser Dissertation zu veröffentlichen und sich mit der nachfolgenden Darstellung meiner und ihrer Mitwirkung an den Publikationen einverstanden erklärt haben.

Ich versichere, dass ich diese Dissertation in allen Teilen selbständig angefertigt habe und dazu keine anderen als die angegebenen Hilfsmittel benutzt habe. Alle aus der Literatur entnommenen Textstellen sind als solche gekennzeichnet.

Die vorliegende Arbeit wurde weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt. Zuvor habe ich keine akademischen Grade erworben oder versucht zu erwerben.

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Structure of the Thesis

This Thesis consists of two chapters, both of which study conditioned and unconditioned gustatory and olfactory behaviour of larval *Drosophila melanogaster*. Initially, I present a general introduction on the chemosensory system of *Drosophila* larvae and a general discussion about the topics of the Thesis. Then, Chapter I provides and develops a behavioural model of interactions between the olfactory and the taste system, focussing on a simple decision whether to behaviourally express an olfactory memory or not. This chapter consists of one published research article and two review articles accepted for publication. Chapter II analyses the processing and various behavioural effects of bitter-tasting substances. This chapter consists of one published research article and one manuscript prepared for publication. Finally, I close the thesis with an outlook on future research.

I take the opportunity to sincerely acknowledge the co-authors of the manuscripts, whose contributions are explicated below. In particular I thank Prof. Bertram Gerber for his supervision and help, without which this work would not have been possible.

Chapter I: Outcome expectations and decision-making in *Drosophila* larvae

Section I.1

Schleyer M, Saumweber T, Nahrendorf W, Fischer B, von Alpen D, Pauls D, Thum A, Gerber B. 2011. A behavior-based circuit model of how outcome expectations organize learned behavior in larval *Drosophila*. *Learn Mem* **18**: 639-653.

	Authors' Initials, contribution from left to right				
Designing the experiments	**MS**	BG	TS	DP	AT
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Analysing and interpreting data	**MS**	BG	TS	DP	AT
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Section I.2

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Creating figures	SD	**MS**	TS	BM

Section I.3

Diegelmann S[=], Klagges B[=], Michels B, **Schleyer M**, Gerber B. In press. Maggot Learning and Synapsin function. *J Exp Biol*

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Chapter II: Taste processing in *Drosophila* larvae

Section II.1

El-Keredy A[=], **Schleyer M**[=], König C, Ekim A, Gerber B. 2012. Behavioural Analyses of Quinine Processing in Choice, Feeding and Learning of Larval *Drosophila*. *PLoS ONE* **7**: e40525. doi:10.1371/journal.pone.0040525

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Section II.2

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Performing the experiments	CK	JL	**MS**	
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Writing	**MS**	JL	BG	CK

Table of Contents

General Introduction and Discussion 8

Chapter I: Outcome expectations and decision-making in *Drosophila* larvae

I.1 A behavior-based circuit-model of how outcome-expectations organize learned behavior in larval *Drosophila*

Abstract 24
Introduction 24
Results 26
Discussion 40
Material and Methods 50
References 57
Supplement 61

I.2 'Decision-making' in larval *Drosophila*

Abstract 66
Introduction 67
Architecture of the chemobehavioral system 68
A working hypothesis of memory trace formation 74
The 'decision' to behaviourally express a memory - or not 82
Aspects of decision-making 87
References 95

I.3 Maggot Learning and Synapsin function

Abstract 100
Behavioural paradigms for associative learning in larval *Drosophila* 101
Chemobehavioural circuitry of larval *Drosophila* 104
Synapsin function: Background 110
Synapsin function: *Drosophila* 113
From memory trace to conditioned behaviour 120
Outlook 123
References 125

Chapter II: Taste processing in *Drosophila* larvae

II.1 Behavioural analyses of quinine processing in choice, feeding and learning of larval *Drosophila*

Abstract 133

Introduction 133

Material and Methods 135

Results 110

Discussion 145

References 150

Supplement 152

II.2 Interaction between bitter and sweet processing in larval *Drosophila*

Abstract 153

Introduction 153

Material and Methods 155

Results 159

Discussion 163

References 169

Supplement 172

Outlook 176

Summary – Zusammenfassung 182

References 186

Curriculum vitae 198

List of publications 199

Thanks – Danke 200

General Introduction and Discussion

In a traditional perspective, animals gather information from their environment to then respond to environmental cues in a way that is adaptive under these circumstances. In other words, the environmental cue drives behavioural change. However, in many cases it is more fruitful to view an animal as an acting subject: When hungry, for example, it is the search behaviour that brings about the food. In other words, behaviour drives environmental change (for a comprehensive overview, see Hoffmann 1993). In this Thesis, I argue that it is both possible and desirable to disentangle responsive and active components of behaviour, and to at least tentatively map them on known features of brain organisation. I do so regarding a rather simple organism, the larva of the fruit fly *Drosophila melanogaster*.

Despite its numerically simple brain and its limited behavioural repertoire, the *Drosophila* larva is nevertheless capable of forming memory traces with surprisingly complex 'content' and embedded in a surprisingly complex network for behavioural organisation (Scherer et al. 2003; Neuser et al. 2005; Gerber and Hendel 2006; Saumweber et al. 2011; reviews: Gerber and Stocker 2007; Gerber et al. 2009). I will first report what is known about odour processing and taste processing in the larva, as well as about the formation and retrieval of odour-taste memories. As I will argue, odour-taste memories are embedded into behavioural organisation in a way that beautifully integrates past, present and future, and that allows the animal to behave according to the expected gain of its actions. It is this quest for gain that drives learned behaviour. Then, I will focus on innate gustatory behaviour with respect to bitter processing and interactions between bitter and sweet pathways. Finally, I will discuss whether innate and learned behaviour are best seen as response or action.

Odour processing

Drosophila larvae sense odours by 21 olfactory sensory neurons within the dome of the dorsal organ (Heimbeck et al. 1999; Fishilevich et al. 2005; Kreher et al. 2005, 2008). Each olfactory sensory neuron typically expresses one type of olfactory receptor (OR) molecule of the *Or* gene family together with the obligatory co-receptor ORCO (formerly known as OR83b) (Fishilevich et al. 2005;

Benton et al. 2006; Pellegrino et al 2011; Vosshall and Hansson 2011). They pass olfactory information to the larval antennal lobe, each of them targeting one of 21 spherical and anatomically identifiable 'glomerulus' compartments (Python and Stocker 2002; Ramaekers et al. 2005).

The information from a given antennal lobe glomerulus is conveyed by typically just one projection neuron (Rameakers et al. 2005; but see Marin et al. 2005) toward two sites: The lateral horn and the mushroom bodies. The mushroom bodies' olfactory input region, the calyx, is organised into approximately 34 glomeruli (Marin et al. 2005; Ramaekers et al. 2005; Masuda-Nakagawa et al. 2005, 2009). The approximately 21 projection neurons signal typically to one calyx glomerulus each and synapse onto approximately 600 Kenyon cells (Lee et al. 1999; but see Technau and Heisenberg 1982). Importantly, there is a fairly stereotyped relation between the antennal lobe glomerulus in which a projection neuron receives its input and the mushroom body calyx glomerulus to which it delivers its output, such that it can be individually identified. A given Kenyon cell collects input from an apparently random draw of up to six (Ramaekers et al. 2005; Masuda-Nakagawa et al. 2005) calyx glomeruli and thus from up to six projection neurons, covering almost a third of the projection neuron coding space. In turn, each projection neuron diverges to 30-180 mushroom body neurons (for details of this approximation see Gerber and Stocker 2007), contributing to up to a third of the mushroom body coding space.

The coding space is drastically reduced in the very next step toward motor output: The Kenyon cells connect to relatively few output neurons (a reasonable estimate may be two dozen; A. Thum, Universität Konstanz, and B. Gerber, LIN Magdeburg, pers. comm.) that link to the motor system *via* an unknown number of synaptic steps (Pauls et al. 2010; for the situation in adults: Ito et al. 1998; Tanaka et al. 2008; Sejourne et al. 2011). These mushroom body output neurons likely receive input from many to all mushroom body cells, thus 'summing up' the activation of the mushroom body.

Importantly, olfactory information reaches the motor systems both *via* the relatively direct lateral horn route and *via* the mushroom body detour. As argued in section I.1 of this Thesis, these two routes organise innate versus learned olfactory behavior.

Taste processing

Compared to the olfactory system, our knowledge about the cephalic 'taste' gustatory system of *Drosophila* larvae is rather fragmentary (reviews on larvae and adults: Gerber and Stocker 2007; Cobb et al. 2009; Montell 2009; Tanimura et al. 2009). It comprises appr. 90 gustatory sensory neurons per body side (Colomb et al. 2007), located in three external (terminal organ, ventral organ, and the bulge of the dorsal organ) and three internal taste organs (ventral, dorsal, and posterior pharyngeal) (Singh and Singh 1984; Python and Stocker 2002; Gendre et al. 2004). All the included gustatory sensory neurons bypass the brain and instead project to particular regions of the suboesophageal ganglion depending on both the receptor gene(s) expressed and their sense organ of origin (Colomb et al. 2007). From the suboesophageal ganglion, taste information is conveyed both to neuromodulatory neurons, including octopaminergic and dopaminergic neurons, projecting toward the brain (Melcher and Pankratz 2005; Colomb et al. 2007; Selcho et al. 2009), as well as directly to (pre-)motor neurons presumably in the ventral nerve cord. However, the details of these connections remain unclear. What is known is that larval behaviour is influenced by tastants that taste sweet, salty or bitter to humans (Hendel et al. 2005; Niewalda et al. 2008; Schipanski et al. 2008; see also Thorne et al. 2004; Montell 2009 regarding adults). In this context, section II.1 studies the behavioural functions of a bitter tastant.

On the molecular level, salt probably is detected by Na^+ channels expressed in the terminal organ (Liu et al. 2003), whereas sugar and bitter detection likely is mediated by G-protein coupled receptors of the *Gr* gene family (Clyne et al. 2000; Dahanukar et al. 2001; Thorne et al. 2004; for review see Montell 2009). Based on *Gr-Gal4* transgene analysis, 39 *Gr* genes (from the 68 genes known in *Drosophila*) have been shown to be expressed in the taste organs of the larval head (Colomb et al. 2007, Kwon et al. 2011). From the known adult-expressed bitter-sensitive GRs (*Gr33a*, *Gr66a*, *Gr93a*) (Scott et al. 2001; Thorne et al. 2004; Jiao et al. 2007; Lee et al 2009), *Gr33a* and *Gr66a* are expressed also in the larva, together with up to 15 other *Gr*s within one cell (Kwon et al. 2011). Strikingly, none of the known adult-expressed sugar-sensitive GRs (*Gr5a*, *Gr64a*, *Gr64f*) (Thorne et al 2004; Dahanukar et al. 2007; Jiao et al. 2007, 2008) is part of those 39 *Gr* genes expressed in the larvae. Research is currently searching for the cellular and molecular basis of sugar perception in

larvae. Lastly, receptors of the *Ir* family (Benton et al. 2009) and/or the *TrpA* family (Kim et al. 2011) may participate in taste perception in the larval stages.

Odour-taste memory formation

Larvae can be conditioned to associate an odour with a gustatory reward (sugar, low concentrated salt) and afterwards prefer the previously rewarded odour over a second, previously non-rewarded odour (Scherer et al. 2003; Neuser et al. 2005; Schipanski et al. 2008). In turn, an odour can be paired with a bitter or highly salty tastant to make larvae avoid the previously punished odour (Gerber and Hendel 2006; Niewalda et al. 2008).

Given the described architecture of the olfactory and gustatory system, how are associations between odour and tastant formed? In 1993, the octopaminergic VUM_{mx1} neuron in the honeybee was identified, which receives its gustatory input likely in the suboesophageal ganglion and provides its output to the antennal lobe, the mushroom body calyx and the lateral horn (Hammer 1993). This single neuron is sufficient to mediate the rewarding effect of sugar in honeybee olfactory learning (this neuron exists in adult [Busch et al. 2009] and larval *Drosophila* as well [Selcho et al. 2012]). Also in *Drosophila* there is evidence that octopaminergic neurons can mediate appetitive reinforcement signalling to connect taste with smell pathways: Adult flies lacking the required enzyme to synthesise octopamine are impaired in odour-sugar learning but not in odour-shock learning. In turn, blocking synaptic output from a set of dopaminergic neurons impairs odour-shock learning but not odour-sugar learning (Schwaerzel et al. 2003). In *Drosophila* larvae, driving sets of octopaminergic/tyraminergic or dopaminergic neurons can substitute for reward or punishment, respectively, in olfactory learning (Schroll et al. 2006). However, the genetic tools used to manipulate octopaminergic/tyraminergic or dopaminergic neurons cover anatomically and functionally heterogeneous sets of neurons. Current research focuses on identifying those individual neurons conferring appetitive or aversive reinforcement signals and telling them apart from neurons mediating other effects, e.g. regarding gustatory processing, olfactory processing, motor control, mediating satiety states or regulation of memory retrieval (Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009, 2012; for adult *Drosophila*: Claridge-Chang et al. 2009; Krashes et al. 2009; Aso et al. 2010, 2012). In this context, it

is important to note that, although the net effect of driving dopaminergic neurons is punishing (Schroll et al. 2006), recent work showed that the so called PAM subset of dopaminergic neurons mediates a reward signal in adults (Liu et al. 2012), suggesting a heterogeneity of different dopamine neurons mediating reward and punishment.

Taken together, it appears to be a reasonable working hypothesis that a tastant, e.g. sugar, activates gustatory sensory neurons that trigger a value signal ('good') likely *via* some of the aminergic neurons (in the case of high-concentration salt or quinine a 'bad' signal is delivered *via* some of the aminergic neurons) and send it to many, if not all Kenyon cells of the mushroom bodies. At the same time, an odour activates a particular pattern of olfactory sensory neurons, leading to the subsequent activation of a particular pattern of glomeruli in the antennal lobe, as well as particular projection neurons and the corresponding Kenyon cells. Conceivably, a memory trace is formed only in that subset of Kenyon cells which are coincidently activated by both odour signal and value signal (Tomchik and Davis 2009; Gervasi et al. 2010). This memory trace is thought to consist of an alteration of the connection between the Kenyon cells and their output neurons. If subsequently a learnt odour is presented, this altered Kenyon cell output is the basis for learned behaviour. Regarding the specific molecular processes at the synapse and especially the role of the presynaptic protein Synapsin, I present a review article I co-authored in section I.3.

The decision whether to behaviourally express a memory – or not

After association of an odour with a reinforcement signal, the learnt odour is able to activate the established memory trace. However, Gerber and Hendel (2006) showed that this activated memory trace is not automatically turned into behaviour: appetitive learned behaviour is seen only in the *absence* of the rewarding reinforcer whereas aversive learned behaviour is seen only in the *presence* of the punishing reinforcer (Gerber and Hendel 2006).

In section I.1, I discuss these findings in detail and then extent them: Especially, after being coincidently exposed to an odour and sugar, *Drosophila* larvae show appetitive learned behaviour when the sugar concentration during training was higher than the sugar concentration at the moment of test, whereas animals tested on a substrate with a sugar concentration equal to or higher than during

training do not. Importantly, learned behaviour is possible in the presence of a high sugar concentration, given that the training-concentration was yet higher. Thus, neither the training concentration *per se* nor the testing concentration of sugar *per se* determines learned behaviour, but their comparison does.

In accord with Gerber and Hendel (2006) I conclude that learned behaviour toward food-associated odours is a *search for food*, which is abolished in the presence of food. This is a fundamentally different perspective from interpreting learned behaviour as *response to the odour*. The larvae compare the value of the activated memory trace with the value of the testing situation and show appetitive learned behaviour depending on the result of this comparison. That is, learned search is behaviourally expressed only if the outcome of tracking down the learned odour promises a gain in the sense of yet-more-reward than is actually present.

After aversive conditioning with quinine, in turn, learned avoidance is seen in the presence but not in the absence of quinine. Such behaviour can be understood as *escape from quinine*, which is pointless in the absence of a reason to escape (the same was found for high-salt [Gerber and Hendel 2006] and mechanical disturbance [Eschbach et al. 2011] as punishment). Thus, learned escape behaviour remains disabled unless such learned escape offers a gain in the sense of relief from punishment. Whether there is a quantitative comparison between memory trace and testing situation like in the appetitive case (see above), however, remains to be directly shown for aversive memories. However, based on experiments using quinine and differently high concentrations of salt, this seems likely (Gerber and Hendel 2006; see discussion in section I.1).

Interestingly, the behavioural expression of a sugar-memory trace is independent of the presence of quinine, and correspondingly the behavioural expression of a quinine-memory trace is independent of the presence of sugar. However, under appropriate conditions the resulting behavioural tendencies certainly can summate: In the presence of quinine, scores from a choice situation between two odours from which one was associated with sugar and the other odour with quinine are higher than after sugar-only training. This is because in the presence of quinine both memory traces can be expressed behaviourally: Learned escape from quinine is expressed because *quinine is present*, and learned search for sugar is expressed because *sugar is absent*.

Thus, learned olfactory behaviour is organised according to its expected outcome, that is toward finding reward and escaping punishment. Referring to the initial question, learned olfactory behaviour therefore is not responsive in nature, but rather is an action expressed for the sake of those things that are not there, the sought-for reward and the attempted relief.

The behavioural organisation described above integrates past experiences, the current situation and the animal's behavioural options. Especially, it entails that the larvae successively form to kinds of expectations: A larva confronted with the choice between two odours is consulting its past experiences in terms of an activated memory trace to predict the expected value of the gustatory environment: If the odour previously was repeatedly paired with sugar, the larva expects to find sugar again. In other words, the larva forms an expectation about how the current situation *should be*. This expectation is pitted against the direct gustatory input of the testing situation (how *it actually is*). Depending on whether the situation is according to the expectation or not, the larva has several behavioural options. Again based on prior experiences, it predicts the consequences of executing each option and finally chooses that option with the best predicted outcome. In other words, the larva forms expectations about what *should happen* if it behaves in a particular way. In case sugar is present, no further action is necessary and the larva behaves irrespective of the previous training. In case no (or too little) sugar is present, the larva searches for more sugar and approaches the odour source that predicts sugar reward.

Importantly, the larva does not find the optimal behaviour by trial and error – but by 'offline' weighing the available options and their consequences. If one wants to put it in other words, the larva takes a decision: Whether to behaviourally express a memory trace – or not. In section I.2, together with Bertram Gerber I discuss in detail the concept of decision-making and which aspects of decision-making can and, maybe more importantly, which ones cannot be studied in larval *Drosophila*.

In contrast to learned olfactory behaviour (processed *via* the mushroom body route of olfactory processing, see above), innate olfactory behaviour (processed *via* the lateral horn route) seems to be more rigid and inflexible: Experimentally naïve larvae are attracted to odours regardless of the gustatory environment. In turn, innate gustatory behaviour toward sugar, salt or bitter is not influenced by the presence of odours, either. I conclude that the learned olfactory processing stream is rather

flexible and open to modifications by the environment, whereas innate gustatory and olfactory processing streams are rather responsive in nature and insensitive to signals from the environment (although we find flexibility of gustatory behaviour in other regards, see below).

Based on the collected data regarding innate and learned behaviour, in section I.1 I suggest a behavioural circuit-model of chemosensory behaviour and the 'decision' process whether to behaviourally express a memory trace or not. This model on the one hand maps nicely to known components of the larval chemobehavioural circuit, and on the other hand provides clear hypotheses about the kinds of architecture to look for in the currently unknown parts of these circuits. In the Outlook of this Thesis I thus propose strategies for future research to further investigate decision-making in *Drosophila* larvae – and its neurogenetic bases.

Bitter processing: from sensory signals to behaviour

In the second chapter, I focus mainly on the animal's needs and its current situation as covered by gustatory perception and processing. Especially, I focus on the processing of bitter tastants. Quinine, the bitter tastant in tonic water and bitter lemon, for example, was known to be aversive to adult (e.g. Meunier et al. 2003; Weiss et al. 2011) and larval *Drosophila* (Hendel et al. 2005). It has been further shown that high concentrations of quinine suppress larval feeding behaviour (Hendel et al. 2005) and, when associated with an odour, can act as punishment (Gerber and Hendel 2006). In section II.1, I confirm these findings together with Amira El-Keredy and extent them with respect to the dose-dependency of all those effects.

In general, animals in this kind of experiments are exposed to chemical substances and their influence on the animals' behaviour is observed. In order to reliably interpret and understand the results of such experiments, a crucial prerequisite is to know how the sensory input from those substances is connected to the behaviours they induce. Unfortunately, our knowledge regarding the organisation of gustatory processing is limited in this regard (see the discussion of section II.1 for a rather detailed overview).

A very simple structure would be that all bitter substances activate one bitter sensory cell (or a homogeneous group of sensory cells) steering all bitter-induced behaviours (Fig.1A). However, recent

data from adults and larvae strongly suggest that there exist heterogeneous subsets of bitter sensory cells: It has been shown by electrophysiology in adults that gustatory sensilla differ in the spectrum of bitter substances they are responding to (Meunier et al. 2003; Weiss et al. 2011). Weiss et al. (2011) identified four classes of bitter-sensitive labellar sensilla (each housing one bitter sensory neuron) based both on the receptor molecules (from the *Gr* gene family) they express and the bitter tastants that activate them. From those four classes, two respond to a small, complementary subset of bitter tastants (the other two classes of sensilla responded to all tested bitter tastants). Although no such data are available in the larvae so far, the organisation of bitter neurons regarding the expressed GR molecules is apparently similar to adults (Kwon et al. 2011). Thus, one would expect a similar organisation on the functional level, too. Anyway, these results suggest that different types of sensory neurons primarily code for the *identity of bitter substances* – at least in a rather rough way (Fig. 1B).

Alternatively, those different types of sensory neurons may primarily code for different *behavioural functions* of bitter tastants (Fig. 1C): In section II.1 we find that the effects of quinine on three examined behaviours (choice, feeding and learning) differ in their dose-response profile. Possibly, therefore, quinine activates heterogeneous sets of bitter sensory neurons steering different behaviours with different gain.

All prerequisites to answer the question of a functional coding within the gustatory sensory cells are now given: Kwon and colleagues provided a library of driver fly strains covering all *Gr* expressing cells in larvae, most of them labelling single cells (Kwon et al. 2011). And in section II.1, we provide a repertoire of behavioural tasks induced by bitter substances. If one transgenetically disables a subset of sensory cells and finds one bitter-induced behaviour to be impaired and another behaviour to be intact, this would prove that different bitter-induced behaviours rely on different sets of sensory cells. Indeed, those types of experiments, focussing on innate *versus* learned behaviour, are currently being performed (A. Thum, Universität Konstanz, pers. comm.), such that one can reckon with new insights into this topic soon.

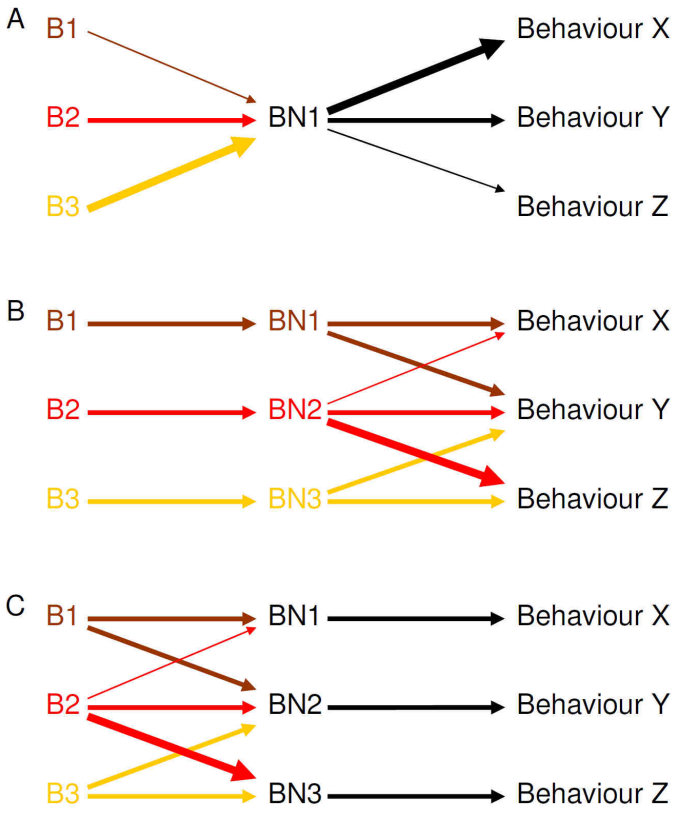

Figure 1: *Three scenarios how bitter sensory input may be converted to behaviour*
(A) As a simple scenario, all bitter tastants (B1-B3) may activate one bitter sensory neuron (BN) or one homogeneous set of neurons that steer all bitter-induced behaviours. Bitter tastants may differ in their ability to activate the BN (indicated by the strength of the lines), and a particular activation of the BN may induce different behaviours to different extents.
(B) Bitter sensory neurons may primarily code the identity of bitter substances. One particular bitter sensory neuron is activated by one particular subset of bitter tastants. Bitter sensory neurons differ in their connectivity to downstream neurons steering behaviours. For example, BN1 (activated by bitter tastant B1), but not BN3 (activated by B3) is connected to behaviour X, thus B1 (*via* BN1) is able to induce this behaviour and B3 (*via* BN3) is not. Furthermore, bitter sensory neurons may form connections to behaviour steering neurons of different strength, thus a given level of activation of BN2 may, for example, strongly induce behaviour Z but only weak behaviour X.
(C) Bitter sensory neurons may primarily code the behavioural function. One particular BN is steering one particular behaviour. Bitter substances differ in their ability to activate this neuron. For example, B1, but not B3 is able to activate BN1 and thus induce behaviour X. Moreover, BNs may differ in their sensitivity to a given bitter substance (due to expressing different receptor molecules or due to different location [e.g. external *vs*. pharyngeal sensilla]), resulting in different dose-behaviour functions induced by, for example, B2.

Bitter-sweet interactions

In the first chapter, I supposed innate olfactory and gustatory behaviour to be rather responsive and hard-wired, being executed without much reference to the environment. In section II.2, however, I find together with Christian König and Judith Leibiger that innate gustatory choice behaviour is influenced by the gustatory environment: The presence of a bitter tastant reduces larval attraction toward sugar. The extent of this effect depends on both fructose and quinine concentration (A similar effect of quinine inhibiting sugar perception and/or processing is known in adults, too [Meunier et al. 2003]). A number of tested sweet tastants differ in their susceptibility to inhibition by quinine, although they induce the same level of attraction. We discuss potential mechanisms of this inhibitory effect, and in the Outlook section of this Thesis I suggest how these scenarios could be tested experimentally.

Thus, although we find no interaction between taste and odour processing in innate behaviour, at least within the taste domain innate gustatory behaviour seems to be open to environmental signals. In

my opinion such flexibility and openness is a prevalent feature of behaviour organisation, reflecting the uncertainty of what will be the best action under any given circumstances. A similar organisation most likely can be found within the innate odour domain, too: Adult flies' attraction toward food-related odours is increased by volatile pheromones from groups consisting of female and male conspecifics but not from groups consisting of only one sex (Lebreton et al. 2012). This finding could hint to super-additive effects of male and female pheromones. Also, it has been reported that the innate aversive response of adult flies toward CO_2 can be inhibited by a particular group of odours found in rotten fruits (Turner and Ray 2009).

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Chapter I:

Outcome expectations and decision-making in *Drosophila* larvae

I.1 A behavior-based circuit model of how outcome expectations organize learned behavior in larval *Drosophila*

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Abstract

Drosophila larvae combine a numerically simple brain, a correspondingly moderate behavioral complexity and the availability of a rich toolbox for transgenic manipulation. This makes them attractive as a study case when trying to achieve a circuit-level understanding of behavior organization. From a series of behavioral experiments, we suggest a circuitry of chemosensory processing, odor-tastant memory trace formation and the 'decision' process to behaviorally express these memory traces- or not. The model incorporates statements about the neuronal organization of innate versus conditioned chemosensory behavior, and the kinds of interaction between olfactory and gustatory pathways during the establishment as well as the behavioral expression of odor-tastant memory traces. It in particular suggests that innate olfactory behavior is responsive in nature, whereas conditioned olfactory behavior is captured better when seen as an action in pursuit of its outcome. It incorporates the available neuroanatomical and behavioral data and thus should be useful as scaffold for the ongoing investigations of the chemo-behavioral system in larval *Drosophila*.

Introduction

Drosophila larvae, being the major feeding stages of the flies' life cycle, have a numerically simple brain, maybe ten million times fewer neurons as compared to man, and possess correspondingly moderate behavioral complexity. These features, together with the general potential of *Drosophila* for

transgenic manipulation (Sokolowski 2001; Elliott and Brand 2008), make them an attractive study case when trying to achieve a circuit-level understanding of behavior, in particular with regard to chemosensory processing and odor-tastant learning (Gerber and Stocker 2007; Gerber et al. 2009).

Drosophila larvae innately ('innate' throughout this paper is used in the sense of: experimentally naïve) show positive preference for sugars (Schipanski et al. 2008) as well as to relatively low concentrations of salt (Miyakawa 1982; Niewalda et al. 2008), but negative preference for high salt concentrations (Liu et al. 2003; Niewalda et al. 2008) and for substances that to humans taste bitter (Hendel et al. 2005; Meunier et al. 2003). Regarding olfaction, larvae are typically attracted to odors but may, for some odors and at high concentrations, also show aversion (Rodrigues 1980; Cobb 1999; Boyle and Cobb 2005; Kreher et al. 2008). Given the numerical simplicity of the chemosensory system in the larva (Heimbeck et al.1999; Ramaekers et al. 2005; Colomb et al. 2007a), a reasonably detailed understanding of innate gustatory and olfactory behavior can be reckoned with (Gerber and Stocker 2007; Kreher et al. 2008; Gerber et al. 2009) (see Discussion).

To complicate matters, however, olfactory larval behavior can be flexible: Larvae can be differentially conditioned to associate one odor with a sweetened reward substrate, and another odor with a not sweetened substrate. After such training, larvae prefer the previously rewarded over the previously non-rewarded odor in a binary choice assay (Scherer et al. 2003; Neuser et al. 2005). Also, by punishing one odor with a bitter or high-concentration salt taste, larvae can be conditioned aversively to odors (Gerber and Hendel 2006; Niewalda et al. 2008).

Presently, the cellular site(s) of these kinds of learning, as well as their molecular mechanisms, are the topic of ongoing research, and one can be hopeful that a comparably detailed picture of these processes can be obtained in the larva as it has been obtained for adult *Drosophila* (Heisenberg 2003; Gerber et al. 2004a, 2009; see Discussion) and bees (Menzel 2001; Giurfa 2007). However, there remains a gap in our understanding of how olfactory memory traces, once established, actually organize behavior, and how innate and learned olfactory behavior are integrated. Notably, the psychological nature of olfactory behavior as response or action is under continued debate: Within cartesian tradition, conditioned behavior often is explained by a change in value of the odor (e.g. Fiala 2007); that is, as result of appetitive training, the odor itself is something 'good' for the animals and

therefore they approach it. In other words, learned olfactory behavior, just as innate olfactory behavior, is regarded as a *response to the odor*.

Alternatively, Gerber and Hendel (2006) (see also Dickinson 2001; Elsner and Hommel 2001; Hoffmann 2003) suggested that it is more fruitful to view appetitive conditioned behavior as an action, taken *in search of food*: Specifically, at the moment of testing the difference between what the animals 'expect' (based on olfactory memory) minus what they 'observe' (based directly on gustatory input) can provide the animals with an estimate of their behaviors' expected gain in terms of finding food. If this expected gain is positive, i.e. if memory promises a situation *better than* the current one, the larva moves towards the previously reinforced odor. Thus, Gerber and Hendel (2006) suggested that it is this expected gain of food, rather than the value of the memory trace *per se*, or of the value of the testing situation *per se*, which is the immediate cause of learned behavior. In an analogous manner Gerber and Hendel (2006) interpreted conditioned aversion as escape behavior. In this case, the expected gain assumes the form of a relief from punishment.

Here, we first ask whether innate gustatory behavior is affected by the presence of odors and whether in turn innate olfactory behavior is affected by the presence of tastants. Regarding associative odor-taste learning, we then ask whether and how learned olfactory behavior is affected by the presence of tastants. We report that learned, but not innate olfactory behavior is affected by the presence of tastants, and propose a minimal, neuroanatomically plausible circuitry that can accommodate the presented behavioral as well as the available neurobiological data. We suggest that the modulating effects that the tastants can exert on learned olfactory behavior at the moment of testing ensures the organization of this learned, but not of innate, olfactory behavior according to its expected outcome.

Results

Experiment 1: Is innate gustatory behavior affected by the presence of odor?

We offer experimentally naïve larvae a choice between two halves of a Petri dish, one filled with pure agarose, the other filled with agarose plus tastant (either 2 M fructose, 5 mM quinine, or 1.5 M salt). Contemplating the time courses of gustatory behavior, which is positive preference with regard to 2

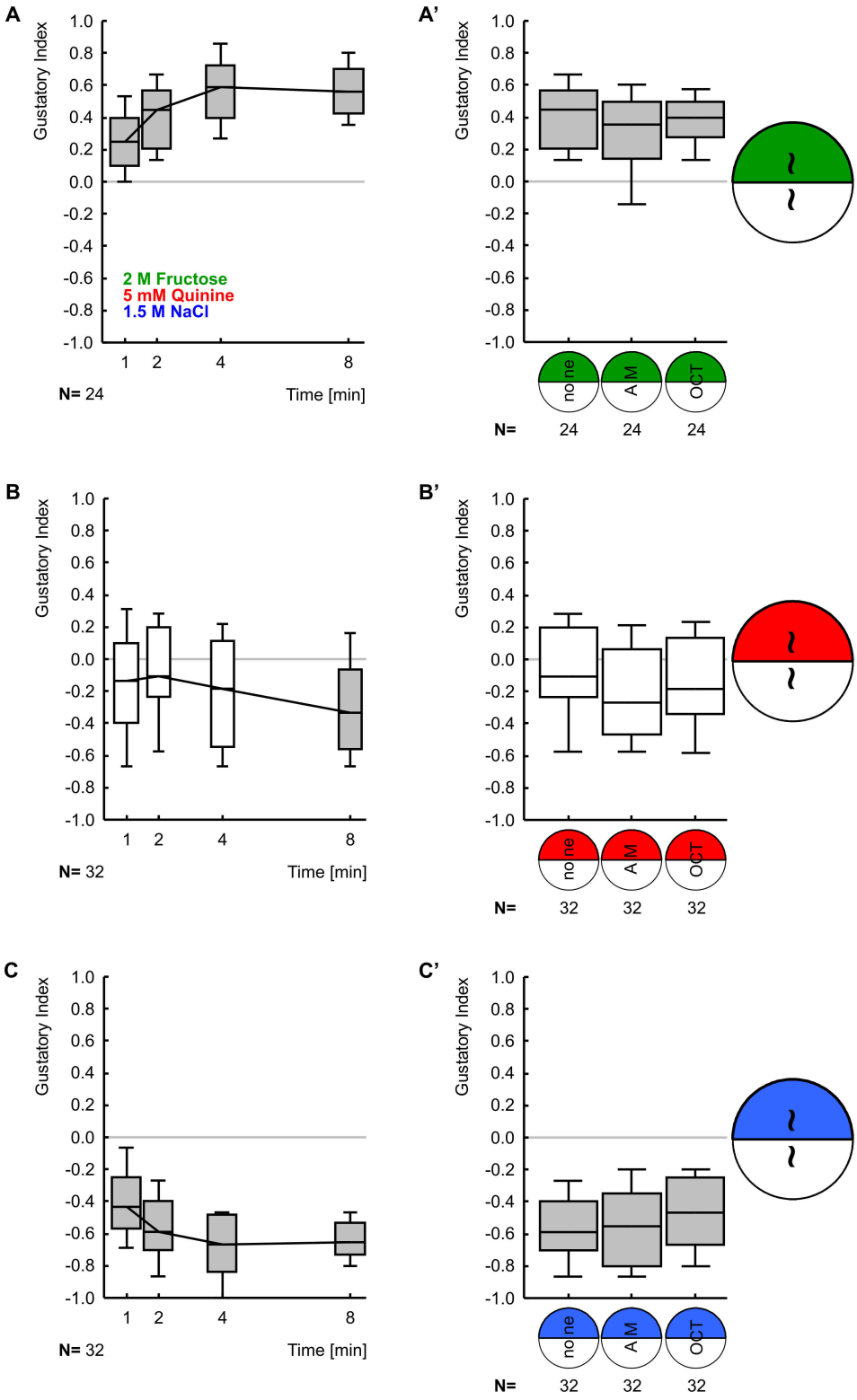

Figure 1 *Is innate gustatory behavior affected by ambient odor?*
Displayed are the tastant preferences towards (A, A') 2 M fructose, (B, B') 5 mM quinine and (C, C') 1.5 M salt. Larvae show positive preference towards fructose and negative preference towards quinine and salt. (A, B, C) show preferences over time, (A', B', C') show preference values after 2 minutes, measured in the presence of either no odor, *n*-amylacetate or 1-octanol. Please note that the 'none' scores in (A', B', C') re-present the '2 min' data from (A, B, C), respectively. The box plots show the median as the bold line, 25 and 75 % quantiles as the box boundaries, and 10 and 90 % quantiles as whiskers. Significant differences from zero ([A, B, C]: $P< 0.05/4$, [A', B', C']: $P< 0.05/3$, one-sample sign tests) are indicated by shading of the boxes.

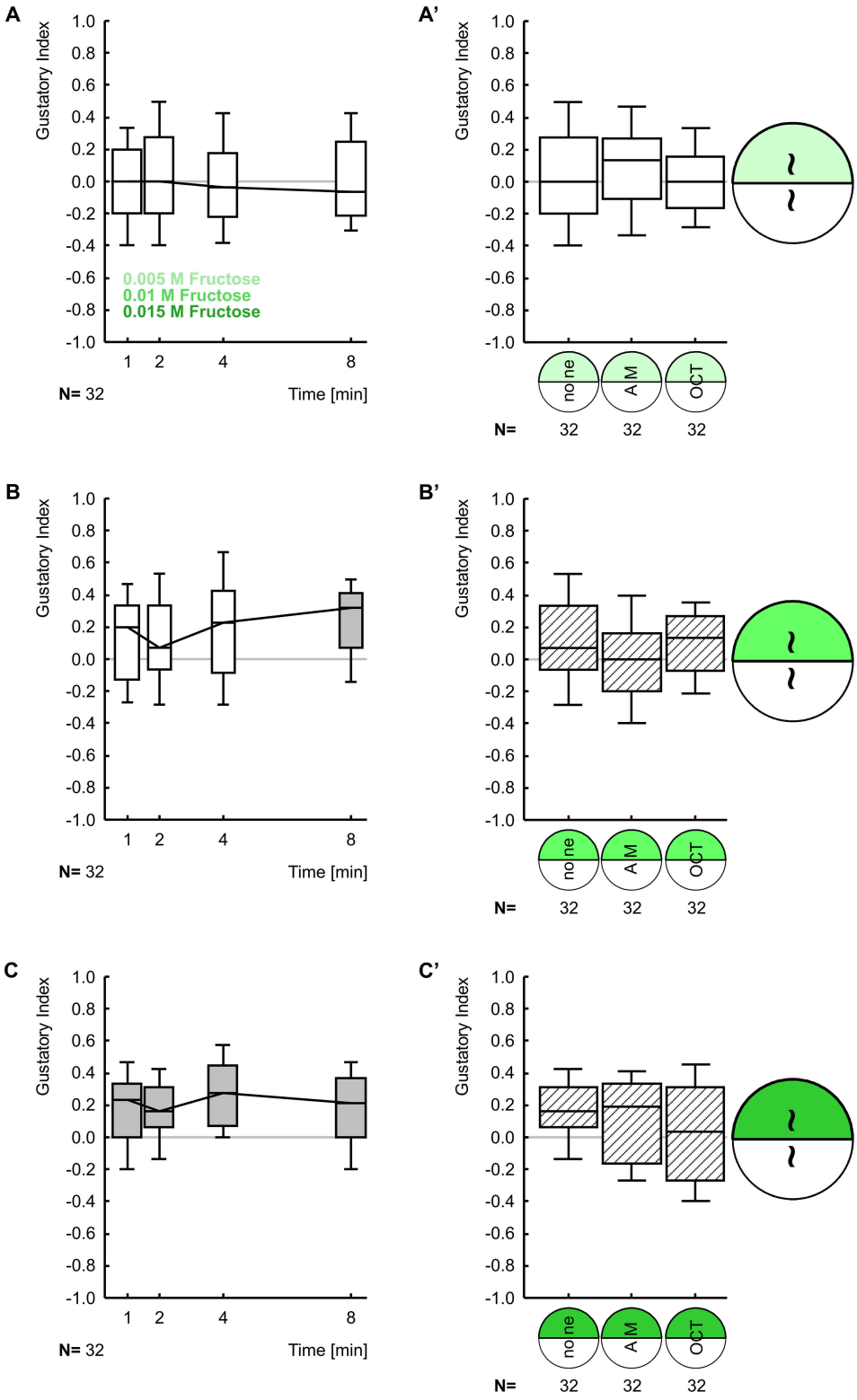

Figure 2 *Do odors affect near-threshold fructose preference?*
Gustatory preference towards (A, A') 0.005 M fructose, (B, B') 0.01 M fructose and (C, C') 0.015 M fructose. (A, B, C) show preferences over time, (A', B', C') show preference values after 2 minutes, measured in the presence of either no odor, *n*-amylacetate or 1-octanol. Please note that the 'none' scores in (A', B', C') represent the '2 min' data from (A, B, C), respectively. For a description of the box plots see legend of Figure 1. Significant differences from zero ($P< 0.05/ 4$, one-sample sign tests) are indicated by shading of the boxes. Hatched shading of the boxes indicates significant differences from zero for the pooled data (B' and C', $P< 0.5/ 3$ in one-sample sign-tests in both cases). Thus, as intended, at the chosen concentration range fructose preference is just around threshold.

M-fructose (Fig. 1A) and negative preference for 5 mM quinine (Fig. 1B) as well as for 1.5 M-salt (Fig. 1C), we choose the 2-min time point (when gustatory behavior has not yet reached its asymptote) to test whether the presence of odor (either *n*-amylacetate diluted 1:50 in paraffin or undiluted 1-octanol) would alter gustatory behavior. This is not the case, neither with regard to fructose (Fig. 1A'), nor quinine (Fig. 1B'), nor salt (Fig. 1C') (Fig. 1: Kruskal-Wallis tests; [A'] H= 1.4, df= 2, *P*= 0.51; [B'] H= 2.9, df= 2, *P*= 0.24; [C'] H= 3.6, df= 2, *P*= 0.16). The same holds true when gustatory behavior is scored at later time points (8 min) (not shown), when tastant-effects are over-all stronger.

Next, following the approach of Shiraiwa (2008), we ask whether behavior towards a fructose concentration which is just-about threshold in the absence of odor (i.e. between 0.005 and 0.015 M; Fig. 2A- C) can be pushed above-threshold by the presence of an odor; this is not the case (Fig. 2: Kruskal-Wallis tests; [A'] H= 0.77, df= 2, *P*= 0.68; [B'] H= 2.9, df= 2, *P*= 0.23; [C']: H= 3.0, df= 2, *P*= 0.22). According to the same rational, we note that odors do not alter near-threshold behavior towards quinine (Fig. 1B'). Regarding salt, we correspondingly seek to take advantage of the fact that behavior towards salt changes from negative preference at high salt concentration towards positive preference as concentration is decreased (Niewalda et al. 2008). The point of draw between these two behavioral tendencies is 0.25 M (Niewalda et al. 2008), which we confirm here (Fig. 3A: One-sample sign-tests; $P > 0.05/4$ in all cases). We reasoned that at this salt concentration our assay should be most sensitive when testing for any modulation by odors: At this concentration, the positive and negative behavioral tendencies of salt just cancel out, so it should be particularly easy to 'tip the balance' towards one or the other kind of behavior. Such modulating effect of odor, however, is not observed (Fig. 3B: Kruskal-Wallis test; H= 0.1, df= 2, *P*= 0.96).

Thus, innate gustatory behavior is 'insulated' against olfactory processing. Tastants therefore appear of inherent, odor-independent value to the larvae; this value is the direct basis for innate gustatory behavior.

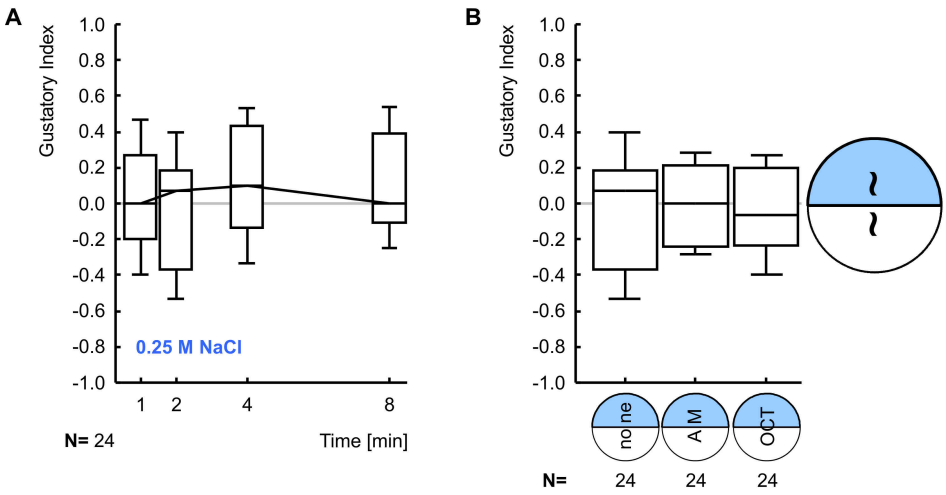

Figure 3 *Do odors tip the balance between attraction and avoidance for salt preference?*
(A) Preferences towards 0.25 M salt in the absence of odor, measured over time. (B) Preference values after 2 minutes in the presence of either no odor, *n*-amylacetate, or 1-octanol. Please note that the 'none' scores represent the '2 min' data from (A). For a description of the box plots see legend of Figure 1. Values of none of the groups are significant different from zero ([A] $P< 0.05/ 4$; [B] $P< 0.05/ 3$, one-sample sign tests) arguing that as intended the attractive and aversive tendencies of salt at this concentration cancel out.

Experiment 2: Is innate olfactory behavior altered in the presence of tastants?

We next ask whether in turn olfactory behavior is modulated by taste processing. Larvae are tested for their choice between an odor-filled container on one side and an empty container on the other side of a Petri dish. This test we perform on either a pure substrate, or on substrates with added fructose (2 M), quinine (5 mM), or salt (either 0.25 M or 1.5 M). We find that neither for *n*-amylacetate (Fig. 4A) nor for 1-octanol (Fig. 4B) olfactory behavior is modified by the substrate condition (Fig. 4: Kruskal-Wallis tests; [A] H= 6.9, df= 4, $P= 0.14$; [B] H= 4.5, df= 4, $P= 0.34$), even when odors are diluted to yield only moderate levels of attraction which arguably are easier to be modulated (Fig. 4: Kruskal-Wallis tests; [C] H= 0.52, df= 4, $P= 0.97$; [D] H= 8.6, df= 4, $P= 0.1$). We notice a small and non-significant tendency of higher attraction towards *n*-amylacetate (diluted 1:50) in the presence of both fructose and quinine, compared to the values obtained on the pure, tasteless substrate (Fig. 4A); the same trend had also been found in previous experiments (data not shown). However, we see this trend neither using a different concentration of *n*-amylacetate (Fig. 4B) nor for using 1-octanol (Fig. 4C, D).

Thus, to the extent tested, innate olfactory behavior seems to be 'insulated' from taste processing. This suggests that odors are of inherent value to experimentally naïve larvae and that this

value, independent of taste processing, is the basis for innate odor attraction. In Figure 11A, we graphically represent this mutual independence between smell and taste behavioral systems.

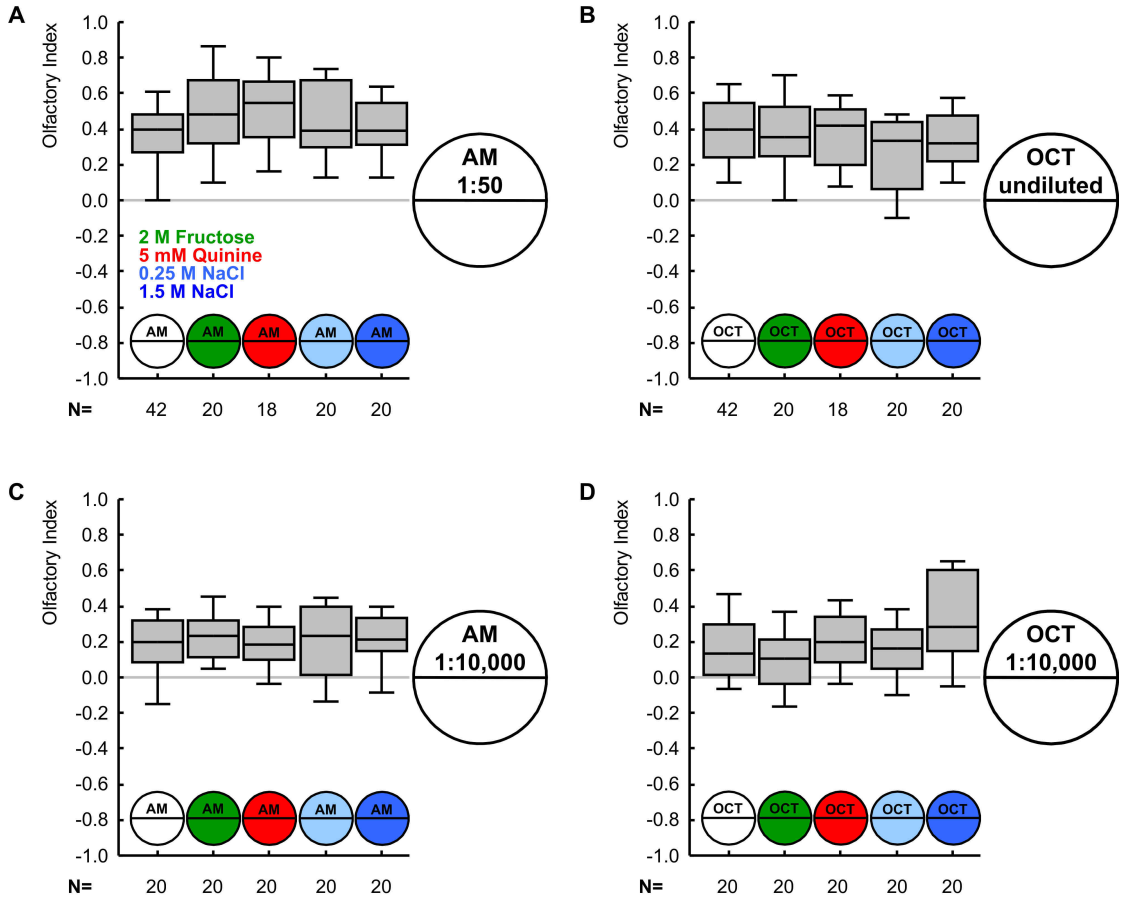

Figure 4 *Is innate olfactory behavior altered in the presence of tastants?*
The Olfactory Index is displayed, measured on the indicated tastant-substrates, regarding (A) *n*-amylacetate diluted 1:50 in paraffin oil, (B) undiluted 1-octanol, (C) *n*-amylacetate diluted 1:10000 in paraffin oil and (D) 1-octanol diluted 1:10000 in paraffin oil. For a description of the box plots see legend of Figure 1. Pooled data of each graph are significantly different from zero ($P< 0.05/ 4$, one-sample sign tests).

Experiment 3: Expected gain drives learned olfactory behavior

The above experiments suggest a mutual independence of innate olfactory and gustatory processing; however, associative training with odors and tastants can modify olfactory behavior (Scherer et al. 2003; Neuser et al. 2005; Gerber and Stocker 2007). Clearly, the formation of an odor-taste memory trace requires an interaction between olfactory processing and a taste-triggered reinforcement signal (Schwaerzel et al. 2003; Schroll et al. 2006) (Fig. 11B; for a discussion see Gerber et al. 2004a; 2009). The following experiments by Gerber and Hendel (2006) had been designed to see whether in addition

there is a second kind of odor-taste interaction, during the translation of such memory traces into conditioned behavior (Fig. 5; for the corresponding Olfactory Index values see Fig. S2). They trained fruit fly larvae to associate an odor either with sugar, quinine, or salt (salt being used at either high, medium, or low concentration; this classification is based on the relative preference between quinine and salt [Fig. S1: Kruskal-Wallis test: H= 178.9, df= 8, $P< 0.05$]). A second odor was always presented without any reinforcer. They then tested for the choice between the two odors in either the absence or presence of that reinforcer which had been used for training. If the training-reinforcer was absent at test (Fig. 5A), larvae behaviorally expressed appetitive memory after sugar as well as after low-salt training; after aversive training with either quinine, high-salt or medium-salt, however, animals did not express any memory (Fig. 5A: Kruskal-Wallis test; H= 26.4, df= 4, $P< 0.05$). If in turn the training-reinforcer was present during test (Fig. 5B), the inverted pattern of results was found: Larvae showed no conditioned behavior in the presence of the appetitive reinforcers, whereas they did show conditioned aversive behavior in the presence of the aversive reinforcers (Fig. 5B: Kruskal-Wallis test; H= 20.9, df= 4, $P< 0.05$). Thus, Gerber & Hendel (2006) interpreted behavior towards previously food-associated odors as search for food, being abolished *in the presence of food*. In turn, fleeing a previously quinine-associated odor is pointless as long as there *is no quinine*.

In a next experiment, Gerber and Hendel (2006) extended these findings (Fig. 6; for the corresponding Olfactory Index values see Fig. S3). Three groups of larvae were trained such that for all groups one odor was presented with quinine, and the other odor with salt. What differed between groups was the concentration of salt, which was chosen as either high, medium, or low. Then, all groups were tested in the presence of quinine (Fig. 6A). Only the groups trained with quinine/ medium salt and quinine/ low salt showed significant conditioned aversion of the quinine-associated odor, whereas the group trained quinine/ high salt did not (Fig. 6A: Kruskal-Wallis test; H= 8.0, df= 2, $P< 0.05$). Therefore, Gerber and Hendel (2006) suggested that memories are behaviorally expressed only if doing so can improve the situation. That is, in the case of training with quinine/ high-salt, quinine was the less bad of the two options (Fig. S1). Therefore, in the presence of quinine, no memory was behaviorally expressed (Fig. 6A). As the salt concentration was reduced, quinine became the worse of the two options (Fig. S1), and hence larvae started to behaviorally express their memory in the presence of

quinine (Fig. 6A). If this reasoning is correct, the pattern of results should be inverted if animals were tested in the presence of the respective salt concentrations. This indeed was found (Fig. 6B: Kruskal-Wallis test; H= 11.2, df= 2, $P< 0.05$) (the fact that, although naïve larvae are indifferent between quinine and the medium salt concentration [Fig. S1], larvae express an avoidance of the salt-associated odor after quinine/ medium salt training [Fig. 6A] may suggest that the learning assay is more sensitive to pick up differences in value between quinine and medium salt processing).

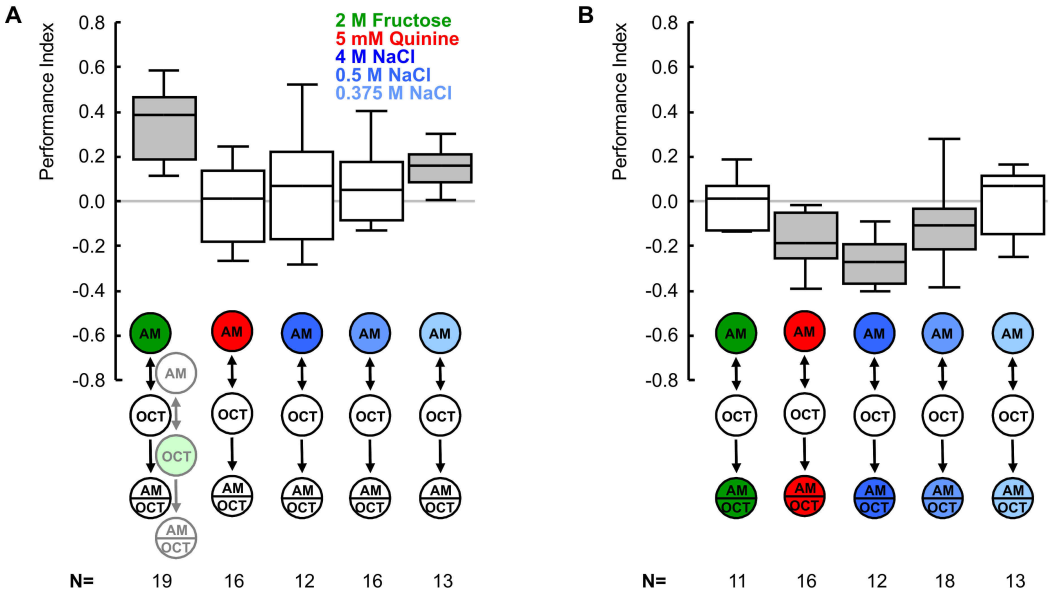

Figure 5 *Expected gain drives learned olfactory behavior (i)*
Behavioral expression of associative memory, as measured by the Performance Index, in (A) the absence or (B) in the presence of the training-reinforcer. Appetitive memories are expressed only in absence, aversive memories only in presence of the training-reinforcer. The sketches below the boxes show the training procedures and test conditions; colored circles represent Petri dishes containing tastant, white circles represent tasteless, pure Petri dishes. For example, in the left-most panel the larvae receive AM with reward and OCT without reward; then, they are tested for their choice between AM and OCT. The reciprocally trained group (dimmed display) receives AM without reward, whereas OCT is rewarded. Fom the difference in preference between the reciprocally trained groups the Performance Index is calculated. Positive Performance Indices indicate appetitive memory, negative values aversive memory. Note that the reciprocally trained groups were run in all cases, but with the exception of the left-most panel are omitted from the sketch for clarity. Also note that in half of the cases the sequence of training trials is as indicated (in the left-most panel e.g. AM+/ OCT and AM/ OCT+), but in the other half is reverse (e.g. OCT/ AM+ and OCT+/ AM). For a description of the box plots, see legend of Figure 1. Significant differences from zero ($P< 0.05/ 5$, one-sample sign tests) are indicated by shading of the boxes.
Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval Drosophila. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. 1).

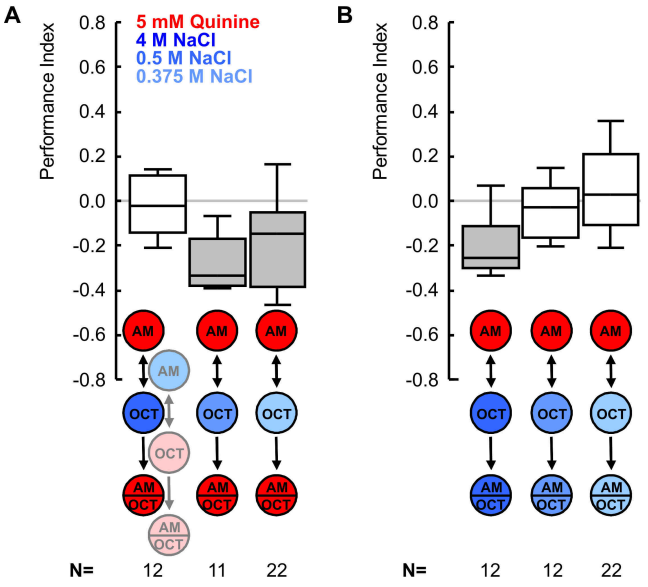

Figure 6 *Expected gain drives learned olfactory behavior (ii)*
All larvae receive one odor paired with quinine, and the other odor with salt. In different groups, the concentration of salt was either high, medium, or low. Testing is performed either in the presence of quinine (A) or in the presence of that salt concentration which had been used for training (B). Memory expression is suppressed if none of the odors predicts a gustatory environment better than the actual test situation. Other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P< 0.05/ 3$, one-sample sign tests) are indicated by shading of the boxes. Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval Drosophila. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. 2).

We here seek to extend these findings to appetitive memory (Fig. 7; for the corresponding Olfactory Index values see Fig. S4). Using the one-odor version of the learning paradigm (see Materials and Methods), four groups of larvae are trained with a medium concentration of fructose (0.2 M) as appetitive reinforcer. The following test is performed either on a pure, tasteless substrate or on a low (0.02 M), medium (0.2 M) or high (2 M) concentration of fructose (Fig. 7: Kruskal-Wallis test; H= 39.1, df= 4, $P< 0.05$). Larvae show conditioned behavior only when the sugar concentration at the moment of test is lower than the sugar concentration during training (One-sample sign-tests; $P< 0.05/ 5$), whereas animals tested on a substrate with a sugar concentration equal to or higher than during training do not (One-sample sign-tests; $P> 0.05/ 5$). Thus, given that the four left-most groups in Figure 7 all are trained the same and consequentially will all have established the same memory trace, it is not the memory trace *per se* that determines the behavior of the animals.

If, in turn, animals are trained with a high concentration of fructose, but are tested in the presence of the medium sugar concentration (Fig. 7; right-most panel), these animals show a higher level of conditioned behavior compared to animals tested on the same medium sweet substrate, but trained with a medium sugar concentration (Fig. 7; Mann-Whitney U-test; U= 28, $P< 0.05$). Thus, also the testing situation *per se* is not a sufficient determinant of appetitive conditioned behavior (this is in contrast to the simple modulation of conditioned behavior by satiety as has recently been investigated

by Krashes et al. 2009). Rather, both the memory trace and the testing situation need to be considered to accommodate learned behavior; specifically, we suggest that the animals compare the value of the activated memory trace with the value of the testing situation and show appetitive conditioned behavior depending on the outcome of this comparison.

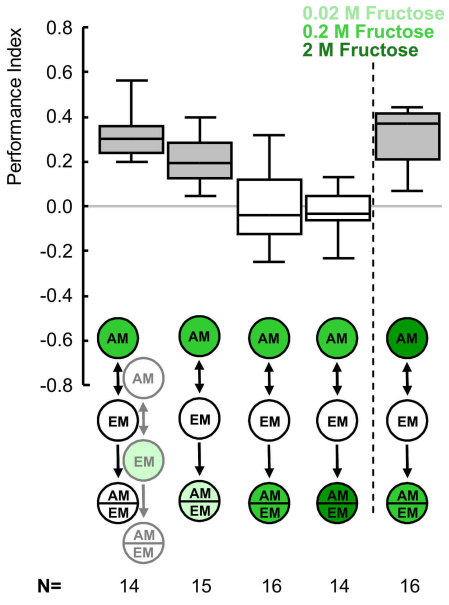

Figure 7 *Expected gain drives learned olfactory behavior (iii)*
Animals are trained using *n*-amylacetate (AM) and empty cups (EM). In all four left-most panels a medium fructose concentration (0.2 M) is used as reinforcer during training; the subsequent test is performed either in absence of fructose, or in presence of a lower-than-trained fructose concentration (0.02 M), the medium training fructose concentration (0.2 M), or a higher-than-trained fructose concentration (2 M). In the right-most panel, a high fructose concentration (2 M) is used during training, but the test is performed in the presence of the medium (0.2 M) fructose concentration. Memory is behaviorally expressed only if the fructose concentration during training is higher than the fructose concentration at the moment of test. Other details as in Figure 5; for a description of the box plots, see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel. Significant differences from zero ($P< 0.05/ 5$, one-sample sign tests) are indicated by shading of the boxes.

Interim Summary

Thus, in contrast to innate olfactory behavior (Fig. 4), learned olfactory behavior is massively influenced by taste processing (Fig.s 5, 6, 7). That is, learned olfactory behavior is not an automatic (Fig. 11B), but rather is a regulated process (Fig. 11C): A comparison between what the animals 'expect' (based on olfactory memory) and what they 'observe' (based directly on gustatory input) can provide them with an estimate of their behaviors' expected gain:

(i) Expected Gain= Expected Value - Observed Value

Learned olfactory behavior requires this expected gain to be positive. In other words, the behavioral expression of a memory trace involves a two-step process: First, the odor activates its memory trace. Second, in an evaluative step, a comparison is made between the value of that memory trace and the gustatory value of the testing situation. If the value of the memory trace for an odor is higher than that

of the gustatory situation, i.e. if there is something to gain, the larva will track down the learnt odor. If the gustatory situation, however, already is as valuable as what the memory trace is promising, conditioned behavior remains suppressed.

Notably, Honjo and Furukubo-Tokunaga (2009), in contrast to the results of Gerber and Hendel (2006) (and also to our findings below), reported that quinine-induced aversive memory can be behaviorally expressed also in the apparent absence of quinine. The authors, however, bathe larvae for 30 min in quinine-solution, which despite extensive washing may induce a lingering bitter after-taste during the test (see also Discussion).

Experiment 4: Independence of appetitive and aversive memory

We next extend the above account by an 18-group experimental design in which larvae are trained differentially using one of three kinds of training regimen:

- One odor is presented with a fructose reward and the second odor without any reinforcement.
- One odor is paired with quinine punishment and the other odor without any reinforcement.
- A push-pull experimental design is used, such that one odor is rewarded and the other punished.

Animals that underwent one of these three kinds of training regimen are then tested for their choice between the trained stimuli in one of three different testing situations: On a tasteless, pure substrate, on a fructose substrate, or on a quinine substrate. Lastly, all experiments are performed using either the two-odor version or the one-odor version of the learning paradigm (see Materials and Methods for details) (Fig. S5 shows the corresponding Olfactory Index scores of all groups of larvae within this experiment).

In keeping with the above account (i), scores after quinine-only training (Fig. 8A, A': Kruskal-Wallis tests; [A] H= 23.9, df= 2, $P< 0.05$; [A'] H= 16.4, df= 2, $P< 0.05$) do not reveal conditioned avoidance of the quinine-associated odor on a pure or on a sweet substrate, thus behavior on both substrates does not differ (Fig. 8A, A': Mann-Whitney U-tests; [A] U= 97, $P= 0.52$; [A'] U= 103, $P= 0.50$). This is because the 'observed' pure and the 'observed' fructose are both better than the 'expected' quinine, such that in both cases the quinine-memory trace is not expressed in behavior. In turn, we observe conditioned avoidance in presence of quinine, different from animals' behavior on

the pure substrate (Fig. 8A, A': Mann-Whitney U-tests; [A] U= 22, $P< 0.05/ 2$; [A'] U= 34, $P< 0.05/ 2$).

Scores after training with reward-only (Fig. 8B, B': Kruskal-Wallis tests; [B] H= 21.7, df= 2, $P< 0.05$; [B'] H= 19.5, df= 2, $P< 0.05$) are higher for the pure test situation than in the presence of fructose (Fig. 8B, B': Mann-Whitney U-tests; [B] U= 14, $P< 0.05/ 2$; [B'] U= 34, $P< 0.05/ 2$), because the 'observed' pure substrate is less valuable than the 'expected' fructose, leading to the behavioral expression of the fructose-reinforced memory trace on the pure substrate. Interestingly, scores are equal in the presence and absence of quinine (Fig. 8B, B': Mann-Whitney U-tests; [B] U= 94, $P= 0.44$; [B'] U= 124, $P= 0.52$), which is somewhat surprising: The above account (i) predicts that the value of quinine, if present at the moment of testing, is offset against the value of a fructose-reinforced memory trace. Thus, conditioned behavior should be expressed particularly strongly when larvae are tested in the presence of quinine after training with fructose, because the difference in value between the 'observed' quinine and the 'expected' fructose is particularly large. This, however, is not observed; we therefore argue that the behavioral expression of a fructose-reinforced memory trace is independent of quinine processing.

Correspondingly, one may ask whether the behavioral expression of a fructose-reinforced memory trace is possible although the behavioral expression of a quinine-reinforced memory trace is suppressed. If this were possible, scores on a pure testing substrate (Fig. 8C, C': Kruskal-Wallis tests; [C] H= 25.3, df= 2, $P< 0.05$; [C'] H= 20.6, df= 2, $P< 0.05$), which allows for the behavioral expression of a fructose-reinforced memory trace but not of a quinine-reinforced memory trace, should be equal after fructose-only and fructose-quinine training. This is indeed what we find (Fig. 8C, C': Mann-Whitney U-tests; [C] U= 112, $P= 0.98$; [C'] U= 114, $P= 0.32$), suggesting that the behavioral expression of the quinine-reinforced memory trace can remain suppressed even if the fructose-reinforced memory trace is in effect.

Given these arguments for independence, we propose separate systems for steering conditioned search and conditioned escape (Fig. 11C, [8, 9]). Within either system, it is determined separately whether conditioned behavior is expressed- or not. That is, conditioned search is expressed if the appetitive memory promises a gain in the sense of yet-more-reward than actually is present:

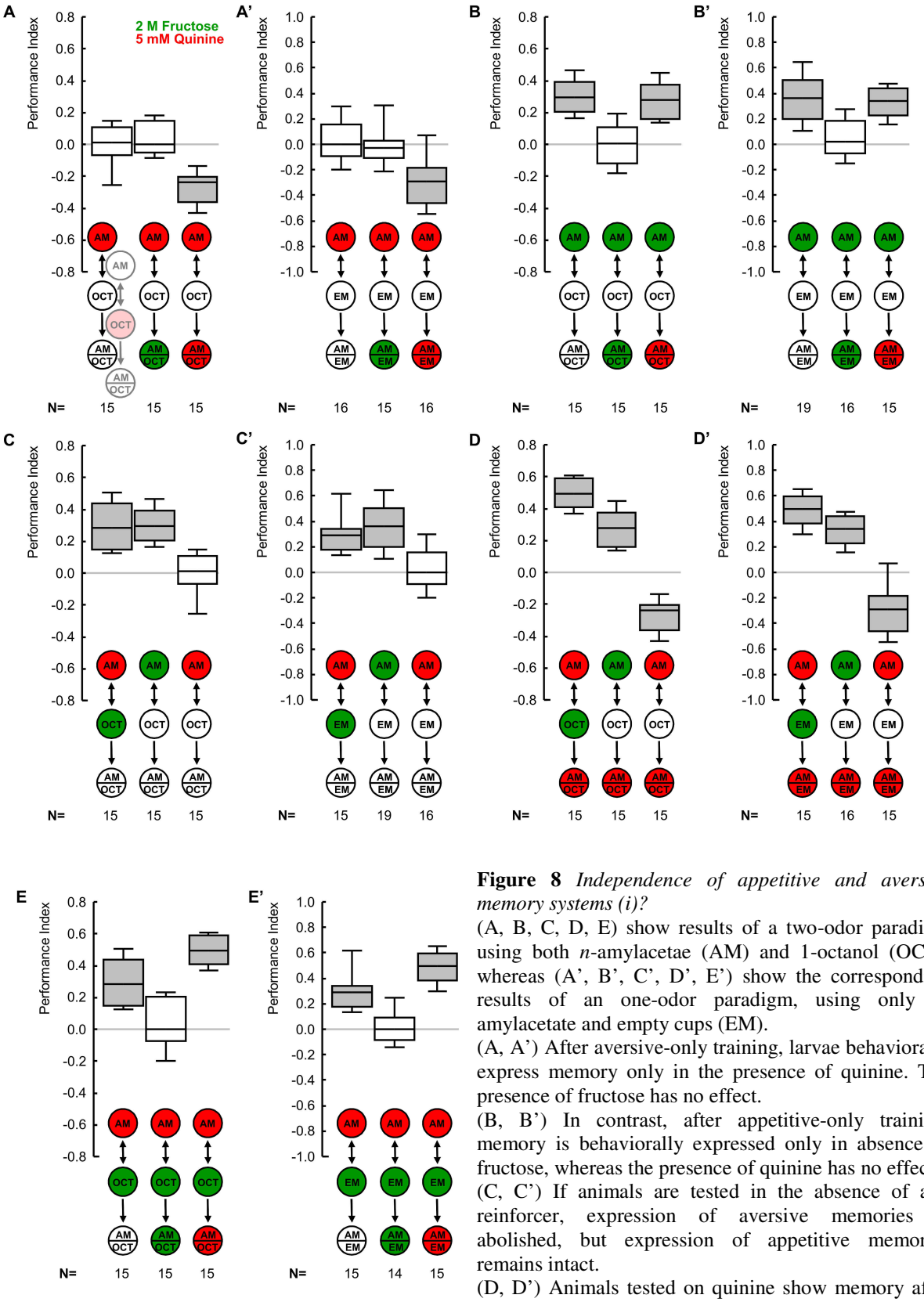

Figure 8 *Independence of appetitive and aversive memory systems (i)?*

(A, B, C, D, E) show results of a two-odor paradigm using both *n*-amylacetae (AM) and 1-octanol (OCT), whereas (A', B', C', D', E') show the corresponding results of an one-odor paradigm, using only *n*-amylacetate and empty cups (EM).

(A, A') After aversive-only training, larvae behaviorally express memory only in the presence of quinine. The presence of fructose has no effect.

(B, B') In contrast, after appetitive-only training, memory is behaviorally expressed only in absence of fructose, whereas the presence of quinine has no effect.

(C, C') If animals are tested in the absence of any reinforcer, expression of aversive memories is abolished, but expression of appetitive memories remains intact.

(D, D') Animals tested on quinine show memory after all kinds of training regimen. Importantly, scores after training in a push-pull regimen using both punishment and reward are higher than those after appetitive-only training, suggesting that both an appetitive and an aversive memory is behaviorally expressed.

(E, E') After push-pull training, scores for animals tested on quinine are higher than for those tested on pure, confirming that only under these conditions both appetitive and aversive memories are behaviorally expressed.

For convenience, some data of this 18-group experiment are included in more than one graph. Other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P < 0.05/3$, one-sample sign tests) are indicated by shading of the boxes.

(ii) Conditioned search if: Appetitive Memory > Observed Reward

Conditioned escape, however, remains suppressed as long as the actual situation is less bad than what aversive memory suggests, i.e. unless escape offers a gain in the sense of relief:

(iii) No conditioned escape if: Aversive Memory > Observed Punishment

This prompted us to ask what will happen if both conditioned search and conditioned escape tendencies are activated. On a quinine substrate, which as we have shown above allows the behavioral expression of both quinine- and fructose-reinforced memory traces, we compare the scores of groups with differing histories of training (Fig. 8D, D': Kruskal-Wallis tests; [D] H= 34.4, df= 2, $P< 0.05$; [D'] H= 34.1, df= 2, $P< 0.05$). Scores in this experiment turn out to be higher after fructose-quinine training than after fructose-only training (Fig. 8D, D': Mann-Whitney U-tests; [D] U= 32, $P< 0.05$; [D'] U= 44, $P< 0.05$). Along the same lines, we find that after fructose-quinine training (Fig. 8E, E': Kruskal-Wallis tests; [E] H= 25.4, df= 2, $P< 0.05$; [E'] H= 26.7, df= 2, $P< 0.05$) scores are higher in the quinine than in the pure testing condition (Fig. 8E, E': Mann-Whitney U-tests; [E] U= 48, $P< 0.05$; [E'] U= 38, $P< 0.05$). We suggest that this is because in the presence of quinine both memory traces can be expressed behaviorally: Conditioned escape from quinine is expressed because quinine *is present*, and conditioned search for fructose is expressed because fructose *is absent*. These two effects can both steer behavior independently of each other, and eventually summate in terms of the distribution of the larvae between the previously rewarded and the previously punished odor.

To further confirm our findings we partially repeat the last experiment using a high concentration sodium chloride (1.5 M; for further details see legends of Fig. 9) instead of quinine (Fig. 9; for the corresponding Olfactory Index values see Fig. S6). In replication of the results of Gerber and Hendel (2006), after punishment-only training with high salt larvae show conditioned behavior when tested in the presence of high salt but not on a tasteless Petri dish (Fig. 9A: Mann-Whitney U-test; U= 3, $P< 0.05$). Notably, after push-pull training with high salt and sugar (Fig. 9B), values are significantly higher when tested on high salt compared to the tasteless test condition (Fig. 9B: Mann-

Whitney U-test; U= 51, $P< 0.05$). Thus, under appropriate testing conditions fructose-induced appetitive and salt-induced aversive memory traces can summate in behavior: On a too salty Petri dish animals both search for sugar and try to escape the high salt concentration.

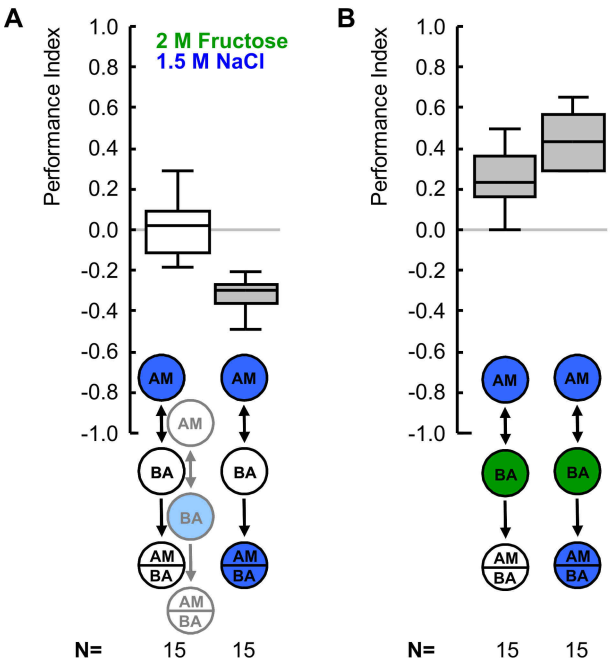

Figure 9 *Independence of appetitive and aversive memory systems (ii)?*
The experiment partially repeats and extends the one shown in Figure 8, using a slightly different protocol: As odors, *n*-amylacetate (AM), diluted 1:250 in paraffin, and undiluted benzaldehyde (BA) are used. Unrewarded, tasteless Petri dishes contain 2.5 % agarose, reward Petri dishes have 2 M fructose added and punishment Petri dishes have 1.5 M sodium chloride added.
(A) Larvae receive aversive training with salt as punishment and are tested either in absence or presence of salt. Larvae show conditioned behavior when tested in the presence of salt but not on a tasteless Petri dish.
(B) After push-pull training with salt punishment and sugar reward, performance indices are higher when tested on salt compared to the tasteless test condition, corresponding to the results of Figure 8.
All other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P< 0.05/ 2$, one-sample sign tests) are indicated by shading of the boxes.

Discussion

We first briefly sketch what is known neurobiologically about the establishment of smell-taste associative memory traces to provide a point of reference for our behavior-based model in the second part of this Discussion.

Associating smell and taste

Larvae can learn to associate an odor with taste reinforcement. This implies convergence between olfactory and taste processing. However, no such convergence has been reported to date: The olfactory system passes on its information from the dorsal organ via only 21 olfactory sensory neurons (Heimbeck et al. 1999; Kreher et al. 2005) to the larval antennal lobe (Fig. 10A), each of them targeting just one of 21 spherical 'glomerulus' compartments (Ramaekers et al. 2005). The information from a given glomerulus is conveyed further by typically just one projection neuron

(Rameakers et al. 2005; but see Marin et al. 2005), connecting to both the lateral horn, a presumed premotor centre, and the mushroom bodies (Python and Stocker 2002). In the mushroom bodies' olfactory input region, the calyx, which is organized into reported 28- 34 glomeruli (Marin et al. 2005; Masuda-Nakagawa et al. 2005, 2009; Ramaekers et al. 2005), projection neurons transmit their signal to several of a total of approximately 600 mature mushroom body neuons (also called Kenyon cells) (Lee et al. 1999; but see Technau and Heisenberg 1982). A given projection neuron innervates only one calyx glomerulus, and a given Kenyon cell collects input from between one to three (Ramaekers et al. 2005) or up to six (Masuda-Nakagawa et al. 2005) calyx glomeruli. The Kenyon cells then connect to relatively few (a reasonable guess may be between one to dozens; Pauls et al. 2010) output neurons that have projections into the lateral horn and other potential premotor centres (Pauls et al. 2010; for the situation in adults: Ito et al. 1998; Tanaka et al. 2008; Sejourne et al. in press). These output neurons likely receive input from many if not all mushroom body cells, thus 'summing up' the total level of activation in their input section of the mushroom body.

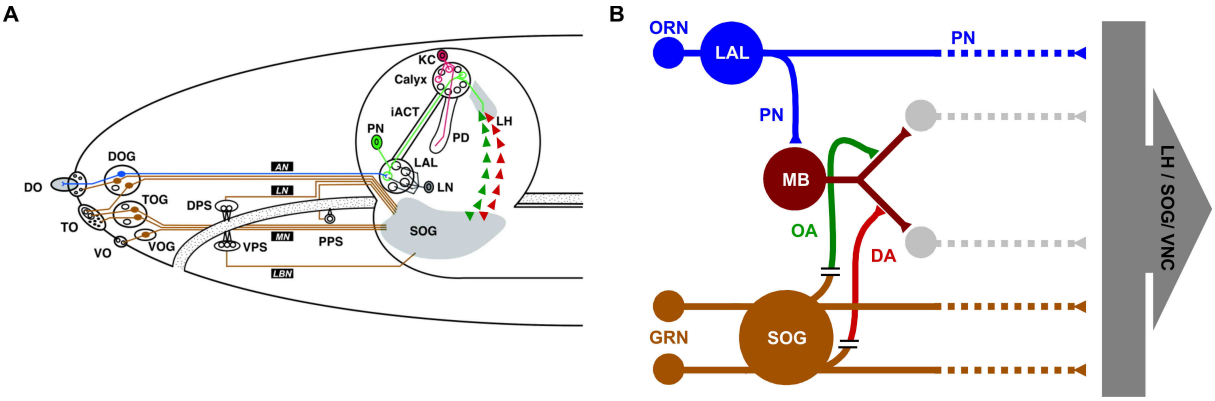

Figure 10 *Neuroanatomy of the larval chemosensory system.*
(A) Schematic diagram of the chemosensory pathways in the larval head. Modified from: Stocker RF. Design of the larval chemosensory system. In: Technau GM, ed. Brain Development in *Drosophila melanogaster*. ©2008 Landes Bioscience and Springer Science+Business Media. (B) Simplified diagram of the chemosensory pathways in the larval brain.
From the three external chemosensory organs, the dorsal organ (DO) comprises both olfactory (the 'dome'; gray) and gustatory sensilla (little circles). The terminal organ (TO), the ventral organ (VO), and the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, PPS) include mainly taste sensilla. The cell bodies of the sensory neurons are collected in ganglia below each sense organ (DOG, TOG, VOG). Olfactory receptor neurons (ORNs, blue) project into individual glomeruli of the larval antennal lobe (LAL), which are interconnected by local interneurons (LN). Projection neurons (PNs, green) carry signals from the LAL to two higher olfactory centers, the mushroom body (MB) calyx and the lateral horn (LH). One intrinsic MB Kenyon cell (KC) is shown in red. Gustatory receptor neurons (GRN, brown) extend to the subesophageal ganglion (SOG). Octopaminergic neurons (OA, green) are proposed to 'short-circuit' a taste-driven reward signal from the SOG toward the MB, dopaminergic neurons (DA, red) carry punishment signals toward the MB. The exact neuronal elements to select particular motor programs when facing tastants and odors are unknown, but likely involve the lateral horn (LH) and ventral nerve cord (VNC).

The gustatory system, in turn, conveys taste information from three external taste organs (terminal organ, ventral organ, and the bulge of the dorsal organ) and three internal taste organs (ventral, dorsal, and posterior pharyngeal organ) (Singh and Singh 1984; Python and Stocker 2002; Gendre et al. 2004), comprising a total of approx. 90 gustatory sensory neurons per body side (Colomb et al. 2007a), to the subesophageal ganglion and then the ventral nerve cord (Fig. 10B; Melcher and Pankratz 2005; Colomb et al. 2007a). Thus, there is no apparent direct connection between smell and taste pathways- consistent with the lack of interaction between innate gustatory and innate olfactory behavior we report in this study.

Given this architecture, the ability of the larva to form an odor-taste associative memory trace may appear surprising. Hammer (1993) in the honeybee identified the octopaminergic VUM_{mx1} neuron, which likely receives gustatory input in the subesophageal ganglion and provides output to the antennal lobe, the mushroom body calyx and the lateral horn. This single, identified neuron is sufficient to mediate the rewarding function of sugar in honeybee olfactory learning (Hammer 1993) (for a description of this neuron in the fly see Busch et al. [2009]; this neuron exists in larval *Drosophila* as well, A.T, unpubl.). As in the bee, also in *Drosophila* there is evidence that at least some octopaminergic neurons 'short-circuit' taste with smell pathways to mediate reinforcement signaling (Fig. 10B): Adult flies lacking octopamine are impaired in odor-sugar learning but not in odor-shock learning. In turn, blocking synaptic output from a subset of dopaminegic neurons impaired odor-shock learning but not odor-sugar learning (Schwaerzel et al. 2003). In larvae, the net effect of driving subsets of octopaminergic or dopaminergic neurons can substitute for reward or punishment, respectively, in olfactory learning (Schroll et al. 2006; this is not at variance with the observation that specific other subsets of these neurons serve different functions, see below). Whether and which of these neurons, in turn, are required for these two forms of learning is less clear (Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009). In any event, important for the current context is that the memory-forming convergence is not between the olfactory and the gustatory pathway itself, but between the olfactory pathway and a modulatory valuation signal ('good' or 'bad', respectively). Such convergence likely happens in the mushroom bodies (Honjo and Furukubo-Tokunaga 2005, 2009; Pauls et al. 2010; Selcho et al. 2009; Michels et al 2011; concerning adults see Riemensperger et al.

2005; Claridge-Chang et al. 2009; reviews by Heisenberg 2003; Gerber et al. 2004a, 2009; concerning honeybees see Hammer and Menzel 1998). If an odor is presented, a particular pattern of olfactory sensory neurons is activated, leading to the activation of a particular combination of glomeruli in the antennal lobe (Kreher et al. 2005), as well as of the projection neurons and the corresponding mushroom body neurons (Masuda-Nakagawa et al. 2005, 2009). At the same time, a tastant, e.g. sugar, activates gustatory sensory neurons that trigger the value signal ('good') *via* e.g. some of the octopamine neurons (in the case of high-concentration salt or quinine: *via* e.g. some of the dopaminergic neurons) and send it to many, if not all Kenyon cells of the mushroom bodies (Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009). Conceivably, only in that subset of Kenyon cells which are activated coincidently by both the odor signal and the value signal, a memory trace is formed (Gervasi et al. 2010; Tomchik and Davis 2009; see also Gerber et al. 2004a for discussion). The memory trace then is thought to consist of a strengthening of connection between the Kenyon cells and their output neurons: If a learnt odor is presented, Kenyon cell output is strong enough to drive the output neurons for triggering conditioned behavior. Indeed, mushroom body output is a requirement for conditioned behavior (regarding adult *Drosophila*: Dubnau et al. 2001; McGuire et al. 2001; Schwaerzel et al. 2003). Following Selcho et al. (2009) and Aso et al. (2010), we stress that the genetic tools available at present to manipulate octopaminergic and dopaminergic neurons, respectively, cover anatomically and functionally heterogeneous sets of neurons. Current research is trying to identify from these sets those neurons conferring reinforcement signaling, and to tell them apart from neurons mediating other effects, e.g. regarding olfactory processing *per se*, gustatory processing *per se*, and signaling of satiety states (Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009; also see Aso et al. 2010; Claridge-Chang et al. 2009; Krashes et al. 2009 for adult *Drosophila*).

Regarding the below discussion, two further aspects should be noted: First, for innate olfactory behavior the mushroom body loop is dispensable (deBelle and Heisenberg 1994), but the projection neurons are required (Heimbeck et al. 2001). This suggests that innate olfactory behavior is supported largely by the direct antennal lobe-lateral horn pathway, whereas conditioned olfactory behavior takes the indirect route *via* the mushroom bodies (see also Saumweber et al. 2011). Second, there is no evidence to argue that a given odor would not activate the same one subset of Kenyon cells during

aversive as well as appetitive learning; this implies that appetitive and aversive memory traces for a given odor may be localized in the same Kenyon cells, but in distinct subcellular compartments (Fig. 11B: [6]) (see discussion in Schwaerzel et al. 2003).

Integrating behavior

Our experimental analyses of chemosensory processing focussed on four kinds of behavior:

- innate taste behavior;
- innate olfactory behavior;
- conditioned olfactory behavior after appetitive learning (conditioned search);
- conditioned olfactory behavior after aversive learning (conditioned escape).

We asked whether the organization of these kinds of behavior is functionally independent of each other.

Clearly, both olfactory and gustatory stimuli support innate behavior. Larvae can show attraction or aversion to odors (Fig. 11A, [1]) (in order to make it easier to relate the behavioral evidence referred to in the text to the diagrams in the Figures, we add the numerals [1-8] to the figure), and show positive or negative preference for tastants, dependent on identity and concentration of the odors and tastants, respectively (Fig. 11A: [2, 3]). We could, despite effort, not find any evidence of interaction between these two pathways: Neither does ambient taste seem to affect olfactory attraction (Fig. 4), nor does in turn ambient odor have an effect on gustatory preference (Fig. 1). The latter may at first sight appear somewhat surprising, as Shiraiwa (2008) had found in adult *Drosophila* that the proboscis extension reflex, an element of feeding behavior, can be facilitated by odors. However, our results certainly do not rule out that odors may, also in the larva, have a potentiating effect on feeding behavior.

In any event, as assayed in this study, the innate locomotor tendencies supported by odors and tastants seem mutually insulated (a situation similar to what we have found for visual and olfactory processing: Yarali et al. 2006). However, the joint presentation of odor and tastant does support the establishment of an associative memory trace, clearly requiring some convergence of both kinds of signalling in the larval brain (also, the joint presentation of visual cues and tastants associatively alters

visual behavior: Gerber et al. 2004b). As discussed above this type of interaction is mediated by modulatory interneurons to 'short-circuit' taste and smell processing, employing distinct sets of neurons to signal reward and punishment (Fig. 11B: [5]). As for a given odor there likely is but one set of Kenyon cells available to enter into association with reward and punishment, these reward and punishment signals likely target different cellular compartments of these cells (Fig. 11B: [6]), from which appetitive and aversive memory traces likely are retrieved via different sets of mushroom body-extrinsic neurons (Fig. 11B: [7]).

In addition to these interactions of olfactory processing and taste-triggered reinforcement signals during training, we identify a second type of interaction. That is, whether these memory traces are behaviorally expressed or not is determined neither by the strength of the memory trace *per se*, nor by the circumstances of testing *per se*, but rather depends on a comparison between the respective memory trace and the value of the test situation: Conditioned search behavior is expressed unless it is *disabled* by the presence of an at least as-good-as-predicted sugar (Fig. 11C: [8]). In contrast, only the presence of quinine or salt at an intensity at least as-bad-as-predicted *enables* the expression of conditioned escape behavior (Fig. 11C: [9]). We would like to stress that these processes require the memory trace to be 'read-out' to allow for this comparison with the value of the test situation; therefore, obviously, these comparisons have to take place downstream of the site of the memory trace. This is critically different from the proposed effect of satiety: Krashes et al. (2009, *loc cit* Fig. 7) suggest that satiety prevents the very read-out of the memory trace, i.e. is acting effectively upstream of the memory trace (site labeled with * in Fig. 11C). Thus, potentially, there may be two mechanisms at operation, one regulating whether a memory trace is addressed and read-out at all (depending on satiety), and another one regulating the behavioral expression of an activated memory trace, dependent on the comparison between memory trace and the testing situation.

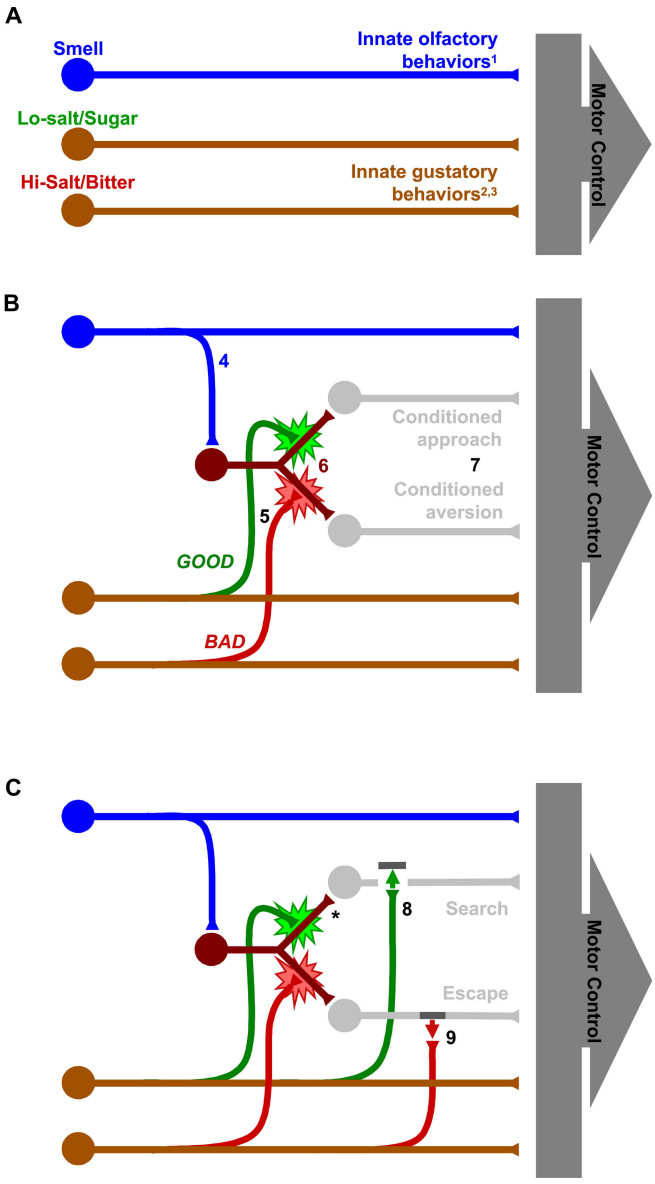

Figure 11 *Behavioral-based circuit of larval chemosensory behavior.*
The figures illustrate, in a sequential way, which kinds of circuitry have to be proposed to accommodate the behavioral experiments reported in this study. To make it easier to relate behavioral evidence referred to in the body text to these diagrams, we add numerals [1-8].
(A) Innate olfactory und innate gustatory behavior are mutually 'insulated' (Fig.s 1, 4). [1] Odors are usually attractive (Fig. 4), except at very high concentrations (e.g. Cobb and Domain 2000; Colomb et al. 2007b). [2] Larvae show negative preference in the case of high concentrations of salt and of quinine (Fig. 1B, C; Hendel et al. 2005; Niewalda et al. 2008) and positive preference in the case of low concentrated salt and sugar (Fig. 1A; Schipanski et al. 2008). [3] We present joint cellular pathways for sugar/ low concentrated salt processing, and for bitter/ high concentrated salt processing, respectively, based on Hiroi et al. 2004. Separated cellular pathways for sugar/ low concentrated salt *versus* bitter/ high concentrated salt are based on Marella et al. 2006.
(B) Establishment of the memory trace and sketch of conditioned olfactory behavior. Larvae can associate an odor with a reward, leading to conditioned approach toward this odor (Fig. 8B; Scherer et al. 2003; Neuser et al. 2005; Schipanski et al. 2008), or with a punishment, leading to conditioned aversion to this odor (Fig. 8A; Gerber and Hendel 2006; Niewalda et al. 2008). Trivially, this requires convergence of the to-be-associated signals. [4] The bifurcation of the olfactory pathway is based on Heimbeck et al. 2001; [5] separated cellular processing of internal reward- and punishment-signals is based on Schwaerzel et al. (2003) and Schroll et al. (2006); [6] separated sub-cellular target regions of internal reward- and punishment-signals are based on the suggestion by Schwaerzel et al. 2003; [7] separated cellular processing of retrieval of aversive and appetitive memory traces is based on Sejourne et al. (in press).
(C) Reconsidering the nature of learned behavior as conditioned search and conditioned escape behavior. Olfactory memory traces are behaviorally expressed only if animals expect to improve their situation: [8] the presence of a reward signal at the moment of testing which is at least as 'good' as predicted blocks the expression of conditioned search behavior (Fig. 8B); [9] in turn, only if at the moment of testing a punishment signal is present which is at least as 'bad' as predicted, conditioned escape behavior is expressed (Fig. 8A).
Please note that the suppressing effect of satiety on appetitive conditioned behavior (Krashes et al. 2009) is proposed to come about by preventing processing beyond the point marked by an asterisk in (C) and thus likely is a process distinct from the one we investigate here.

Generality?

As discussed above, we propose that animals express an aversive memory if they are motivated to escape from the test situation. Presenting an aversive reinforcer, as we did in our experiments, may not be the only way to induce such motivation. Indeed, there exist a broad range of studies on aversive

conditioned behavior in insects that do not report the necessity of an apparent aversive reinforcer at the moment of test. Arguably, however, in these cases there may exist other sources of escape motivation:

Honjo and Furukubo-Tokunaga (2009) used a non-reciprocal quinine-learning paradigm and found aversive memory scores in the absence of quinine. However, as in that study animals were bathed in a liquid quinine solution during training, there may be a lingering bitter after-taste at the moment of test. That is, although no quinine is presented on the test Petri dish, animals may still regard the substrate as unpleasantly bitter and seek to escape from it. Such a lingering taste may also be an explanation for the reported differences in stability of appetitive and aversive memory (half-maximal effects after 90 and 10 minutes, respectively: Honjo and Furukubo-Tokunaga 2005, 2009): with the lingering taste subsiding, scores of conditioned aversion quickly decay.

Regarding larval electroshock-learning, Pauls et al. (2010) report that associative aversion scores are more negative if the last training trial included electric shocks, compared to animals that received electric shock in the previous-last training trial. This again may hint at a residual effect of electric shock that may motivate the animals' escape during test, which may be stronger for those groups that had received shock just prior to testing (in the related study by Khurana et al. [2009] data were not analyzed regarding sequence effects).

Similar arguments may apply in the case of olfactory electroshock learning in adult flies (Tully and Quinn 1985), especially as the intensity of electric shocks in this paradigm is often close to causing physical damage. Interestingly, Tempel et al. (1983) report that aversive memory scores induced by electroshocks are much less stable than appetitive memory scores induced by sugar (half-maximal effects are found after 2 and 18 hours, respectively). Again, this may hint at a carry-over effect of the aversive reinforcer that vanishes after training, such that the driving force behind associative aversion may get lost. Also, before the arms of the T-maze are opened in this type of assay, about one hundred fairly stressed flies are crowded in a volume approximately as small as a cherry (appr. 1.5 cm^3), a situation that should be unpleasant to the flies. Along the same lines, the training and testing situation in sting-extension reflex conditioning of honeybees fixates the animals horizontally on their backs (Giurfa 2007, *loc cit* Fig. 1), which may not be a leisurely body posture for them.

On the other hand, after conditioning crickets with saline solution (Matsumoto and Mizunami 2002, *loc cit* Fig. 3) animals avoid the punished odor in a not apparently unpleasant situation. This observation seems to not fit to the rule of escape motivation suggested above. We note, however, that in this paradigm odor and punishment are presented not only in very close temporal but also in very close spatial proximity, potentially prompting the odor to stand-in for the punishment, rather than becoming a signal-for punishment. A similar argument may apply to odor-taste learning in *Spodoptera littoralis* larvae (Salloum et al. 2011).

Thus, we hesitate to judge whether the behavioral organization of learned behavior as found in this study is an exceptional case or whether it reveals a principle that had remained opaque in previous assays that may have "implicitly" provided a bad-enough testing situation. Indeed, in a recently developed paradigm of association between odor and mechanosensory disturbance as punishment in larval *Drosophila*, learned behavior likewise is only revealed in the presence of that punishment (Eschbach et al. In press). Also, Schnaitmann et al. (2010), analyzing visual learning in adult flies, report that after punishing animals with formic acid during training, aversive memory is behaviorally expressed in the presence but not the absence of formic acid (*loc cit* Fig. 10). Importantly, in this paradigm 50-100 flies can freely move about a large, 9 cm diameter test arena, such that there may not be any reason to escape unless "explicitly" provided by the experimenter- by adding formic acid to the test situation. Similarly, flies trained to associate visual landmarks with a comfortably cool spot in an otherwise uncomfortably hot arena search at the trained location if the testing arena is uniformly hot (Ofstad et al. 2011), but not nearly as well when it is uniformly cool (M. Reiser, HHMI JFRC, pers. comm.).

Outlook

Contemplating the neuronal architecture of the insect olfactory system (Fig. 10; for a recent review: Galizia and Rössler 2010), one of the striking features is that antennal lobe output has two target areas, the mushroom bodies and the lateral horn. These, we propose from our behavior analysis (Fig. 11), correspond to one flexible, open processing stream, and one more rigid, closed one:

- Along the mushroom body route, olfactory processing is integrated with at least two kinds of gustatory signal, namely a reinforcement signal to induce associative plasticity in the mushroom bodies, and a value signal regarding the current status of the gustatory environment. When encountering a conditioned odor, this 'triadic' architecture accommodates a regulatory step, an element of 'pondering' if you will, between the activated memory trace and behavior control: It integrates the past experience of the larva (in the form of the memory trace activated by the odor), its present matter of concern (in terms of the present gustatory environment), and its options for future action (in terms of the premotor neurons for conditioned behavior). This endows the animal with the option to express conditioned olfactory behavior- or not. The flexibility and openness of this architecture, we suggest, is a basic feature of behavior organization, reflecting the fundamental uncertainty in the world as we find it in general, and the uncertainty of what will be the best action under any given set of circumstances in particular.
- In contrast, the direct antennal lobe-lateral horn pathway is relatively rigid and closed: It is effectively 'insulated' against gustatory processing (as well as against visual processing: Yarali et al. 2006). There are few if any degrees of freedom along this processing stream, such that a given olfactory stimulus is, without much reference to what goes on in the 'rest' of the brain, able to organize behavior. Such relatively hard-wired organization, we argue, reflects the outcome of evolutionary trial and error, a phylogenetic curbing of the initially open and flexible organization of behavior to those few odor-behavior relationships that fit under almost all circumstances.

Considering the contrast to the relatively rigid, closed processing stream along the antennal lobe-lateral horn pathway, we suggest that conditioned olfactory behavior organized along the mushroom body loop assumes characteristics of a 'decision' (Zhang et al. 2007), in our case in the sense that conditioned behavior can be expressed- or not. Such a 'decision' appears simple enough to allow experimental access- and complex enough to remain interesting.

For now, the proposed functional circuitry is merely a working hypothesis, a scaffold to investigate the cellular sites of associative plasticity, the tastant-signals modulating, as well as the downstream motor effectuators organizing learned olfactory behavior. It should thus, we hope, bring

us closer to a comprehensive understanding of what makes a larva do what a larva ‘s got to do- and to the implementation of this understanding into a bio-inspired robot.

Materials and Methods

General

Drosophila melanogaster of the Canton-S wild-type strain are used and kept in mass culture, maintained at 25 °C, 60-70 % relative humidity and a 14/ 10 h light/ dark cycle. Experiments are performed under a fume-hood at 20- 24 °C room temperature and use five-day old feeding-stage larvae collected from the food slurry and gently washed in tap water before the start of the experiments. Petri dishes used (Sarstedt, Nümbrecht, Germany) are of 85 mm diameter (except in the case of the experiments displayed in Figures 1- 3, which use 52-mm Petri dishes); they are prepared freshly the day before experiments and contain solidified 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany) (only the experiment displayed in Fig. 9 uses 2.5 % agarose). As the respective experiments require, tastants (fructose [FRU; CAS: 57-48-7; purity 99 %; Roth, Karlsruhe, Germany]; quinine hemisulfate [QUI; CAS: 6119-70-6; Sigma-Aldrich, Seelze, Germany], or sodium chloride [NaCl; CAS: 7647-14-5; purity 99.5%; Roth, Karlsruhe, Germany]) are added to the agarose at the respectively indicated concentrations to create sweet, bitter or salty substrates. Odors (*n*-amylacetate [AM; CAS: 628-63-7; Merck, Darmstadt, Germany]; 1-octanol [OCT; CAS: 111-87-5; Sigma-Aldrich, Seelze, Germany]; benzaldehyde [BA; CAS: 100-52-7; Fluka, Buchs, Switzerland]) are presented by custom-made Teflon containers with 5 mm diameter, covered by a lid with seven 0.5-mm holes as soon as 10 ml of odor has been loaded; dilutions are made in paraffin oil (CAS: 8012-95-1; Sigma-Aldrich, Seelze, Germany). Only the experiments displayed in Figures 1- 3 use another way of odor application (see below).

Is innate gustatory behavior affected by ambient odor?

For assaying innate gustatory behavior, 52-mm diameter Petri dishes are divided into two halves by using a vertical barrier cut from overhead transparencies (Hendel et al. 2005). One half of the dish is filled with only solidified 1 % agarose and the other with agarose in addition containing either of three tastants (FRU, QUI, NaCl). Shortly before the substances solidify, the barrier is removed and Petri dishes stored for use on the following day.

Larvae are put in the middle of such a split Petri dish. After 1, 2, 4 and 8 minutes their location is repeatedly determined as either on the tastant side ($\#_{TASTANT}$), on the agarose-only side ($\#_{PURE}$) or in an approximately 1-cm wide 'middle' stripe of the plate ($\#_{MIDDLE}$; for clarity, this middle stripe is not displayed in the sketches of the Figures) as well as the total number of larvae ($\#_{TASTANT} + \#_{PURE} + \#_{MIDDLE} = \#_{TOTAL}$). Given that for these experiments the small-diameter Petri dishes are used, and that we repeatedly score larval behavior at fairly short intervals, only fifteen larvae are used in all gustatory behavior assays. From these data, a Gustatory Index (GI) [-1; 1] is calculated as:

(iv) $GI = (\#_{TASTANT} - \#_{PURE})/ \#_{TOTAL}$

Thus, positive values indicate positive preference to the tastant, negative values negative preferences to the tastant.

To test whether innate gustatory behavior is altered by the presence of an odor, the Gustatory Index is determined (a) in the absence of odor, (b) in the presence of *n*-amylacetate (AM; diluted 1:50) or (c) in the presence of 1-octanol (OCT; undiluted).

To prevent an accumulation of the larvae at and immediately around the odor containers, in only this experimental series no odor containers are used. Rather, two 0.5 cm x 0.5 cm filter papers are attached to the inner side of the lid of the Petri dish, each on one side of the plate, using double faced adhesive tape. Filter papers are loaded either both with 5 µl of AM or both with 5 µl of OCT. As control condition, no odor is added to the filter papers.

Is innate olfactory behavior altered in the presence of tastants?

To test whether, in turn, innate olfactory behavior is influenced by the presence of tastants, behavior of experimentally naïve larvae toward odor is assayed on a Petri dish containing either (a) agarose-only or on Petri dishes which in addition contain (b) fructose (2 M), (c) quinine (5 mM), (d) low-salt (0.25 M), or (e) high-salt (1.5 M).

Petri dishes (85 mm diameter) are filled with either 1 % solidified agarose or with agarose plus an added tastant and are used the following day. Two Teflon containers are placed at the circumference of the Petri dish, on opposing sides; one is loaded with 10 µl of odor (either AM or OCT, at the respectively indicated dilution), while the other container serves as control with no odor added (empty, EM). For all olfactory behavior assays, the large-diameter Petri dishes are used to yield sufficient distances for odor gradients to form. As in addition no temporal resolution of larval behavior is attempted, all olfactory behavior experiments, including all learning experiments, use groups of thirty larvae. Larvae are transferred to the middle of Petri dish; after 3 min, we determine the number of animals at the odor side ($\#_{ODOUR}$), the number at the no-odor side ($\#_{EM}$) as well as in a 1-cm wide middle stripe ($\#_{MIDDLE}$; for clarity, this middle stripe is not displayed in the sketches of the Figures), and the total ($\#_{ODOUR} + \#_{EM} + \#_{MIDDLE} = \#_{TOTAL}$) number of larvae and calculate an Olfactory Index (OI) [-1; 1] as:

(v) $OI = (\#_{ODOUR} - \#_{EM})/ \#_{TOTAL}$

Thus, positive values indicate attraction to the odor, negative values aversion.

Conditioned olfactory behavior: Two-odor paradigm

Three kinds of training are used: (a) appetitive training, (b) aversive training, or (c) a 'push-pull' combination using both reward and punishment. For appetitive training, larvae receive either of two training protocols: Either AM is presented with reward and OCT without reward (AM+/ OCT), or they are trained reciprocally (AM/ OCT+). For aversive training, the procedure is analogous (AM-/ OCT or AM/ OCT-). For the push-pull experimental design, one odor is rewarded and another odor is punished (AM+/ OCT- or AM-/ OCT+). In all cases, we measure the choice between AM *versus* OCT

in a final test. As reward and punishment, respectively, we use fructose, quinine or salt added to agarose, in the concentrations mentioned in the Results section.

Specifically, two odor containers are loaded with odor (unless mentioned otherwise, either with AM diluted 1:50 in paraffin oil, or with undiluted OCT) (diluting AM ensures that innate responses to AM and OCT are about equally strong [compare Figures 4A and 4B]) and placed onto a Petri dish that either does or does not contain a tastant-reinforcer. These two containers (both loaded with the same odor) are placed at the outer circumference of a Petri dish, on opposing sides. For the first training trial, larvae are transferred to the Petri dish; after 5 min they are transferred to a fresh dish with the alternative odor-substrate combination for the second training trial. For example, during appetitive training larvae are first exposed to AM in the presence of fructose (AM+), and then to OCT in the absence of fructose (OCT) (AM+/ OCT training). This training cycle is repeated three times. Then, animals are placed in the middle of a Petri dish with AM on one side and OCT on the other. This test plate may or may not contain a tastant-reinforcer, as is mentioned along the Results section.

After 3 min, we determine the number of animals at the AM side ($\#_{AM}$), the number at the OCT side ($\#_{OCT}$), the number of larvae on the middle stripe ($\#_{MIDDLE}$) and the total ($\#_{AM} + \#_{OCT} + \#_{MIDDLE} = \#_{TOTAL}$) number of larvae and calculate an Olfactory Index (OI) [-1; 1] as:

(vi) $OI = (\#_{AM} - \#_{OCT})/ \#_{TOTAL}$

Then, a second group of larvae is trained reciprocally, such that OCT is rewarded and AM is not (AM/ OCT+). From these alternately run, reciprocally trained groups we calculate a Performance Index (PI) [-1; 1] as:

(vii-a) $PI = (OI_{AM+/\ OCT} - OI_{AM/\ OCT+})/ 2$

Thus, positive PIs indicate appetitive, negative values aversive conditioned behavior.

In half of the cases the sequence of training trials is as indicated in the previous example (i.e. AM+/ OCT and in the reciprocal group AM/ OCT+), and in the other half of the cases the sequences

are reversed (i.e. OCT/ AM+ and in the reciprocal OCT+/ AM). Notably, the sequence of training trials does not have an effect on behavior at test (Schleyer 2009; Saumweber et al. 2011), and hence the assignment of data for the calculation of the PI is unproblematic (see also appendix of Hendel et al. 2005). For aversive training and push-pull-training, training is performed in an analogous way and the Performance Indices are calculated respectively as:

(vii-b) $PI = (OI_{AM-/\,OCT} - OI_{AM/\,OCT-})/\,2$ after aversive training

(vii-c) $PI = (OI_{AM+/\,OCT-} - OI_{AM-/\,OCT+})/\,2$ after push-pull training

Conditioned olfactory behavior: One-odor paradigm

In order to confirm our findings we repeat the kinds of experiment described above in a number of cases, but using only one odor. That is, training and test are run in the very same way as described, but OCT is omitted throughout, such that instead of loading the respective container with OCT, an empty container (EM) is used. Thus, appetitive training follows the logical structure of training as either AM+/ EM or in the reciprocal AM/ EM+. Aversive training is run as either AM-/ EM or in the reciprocal as AM/ EM-, and the push-pull version uses either AM+/ EM- or AM-/ EM+ training. Again, the sequence of trial types is reversed in half of the cases (see above). Then, larvae are tested for their choice between AM and EM on the respectively mentioned type of substrate and data are analyzed as detailed above.

Data analysis

Given that behavioral data typically are not normally distributed (and in particular as data within restricted intervals by definition are not normally distributed), non-parametric statistics (one-sample sign test, Kruskal-Wallis test, Mann-Whitney U-test) are applied throughout, using Statistica 7.1 (StatSoft, Tulsa, USA) for the PC (the one-sample sign-test uses a web-based statistic tool provided on http://www.fon.hum.uva.nl/Service/Statistics.html). When multiple one-sample or pair-wise comparisons are made within an experiment, a Bonferroni correction keeps the experiment-wide error rate below 5 % by dividing the critical *P*-value by the number of tests (e.g. for three tests $P < 0.05/\,3$);

this is a conservative approach to significance-testing. Data are displayed as box plots, where the middle line shows the median, the box boundaries the 25, 75 % quantiles, and the whiskers the 10, 90 % quantiles.

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Supplement

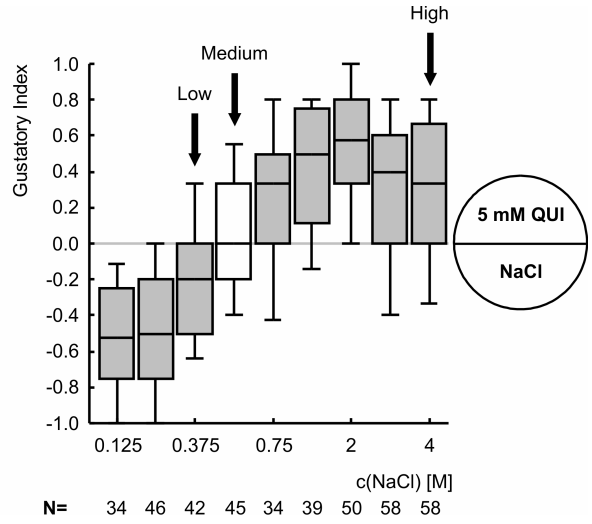


Figure S1 *Choice between QUI and salt.*
Innate relative preferences of larvae between 5 mM quinine and various concentrations of salt. One half of a split Petri dish is filled with agarose containing 5 mM quinine solution (QUI) and the other half with agarose containing the indicated concentration of NaCl (SALT). A relative Gustatory Index (GI_R) [-1; 1] is calculated as:
(S-i) $GI_R = (\#_{QUI} - \#_{SALT})/ \#_{TOTAL}$
Thus, positive values indicate that animals prefer quinine, negative values indicate they prefer salt. Salt concentrations are classified as 'low' if the animals prefer the salt side, and as 'high' if they prefer the bitter side. As the larvae are indifferent when facing the choice between quinine and 0.5 M salt, this concentration is classified as 'medium'. The concentrations of salt used in Figures 5 and 6 are indicated by arrows. For a description of the box plots see legend of Figure 1. Significant differences from zero ($P< 0.05/ 9$, one-sample sign tests) are indicated by shading of the boxes.
Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval Drosophila. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. S1).

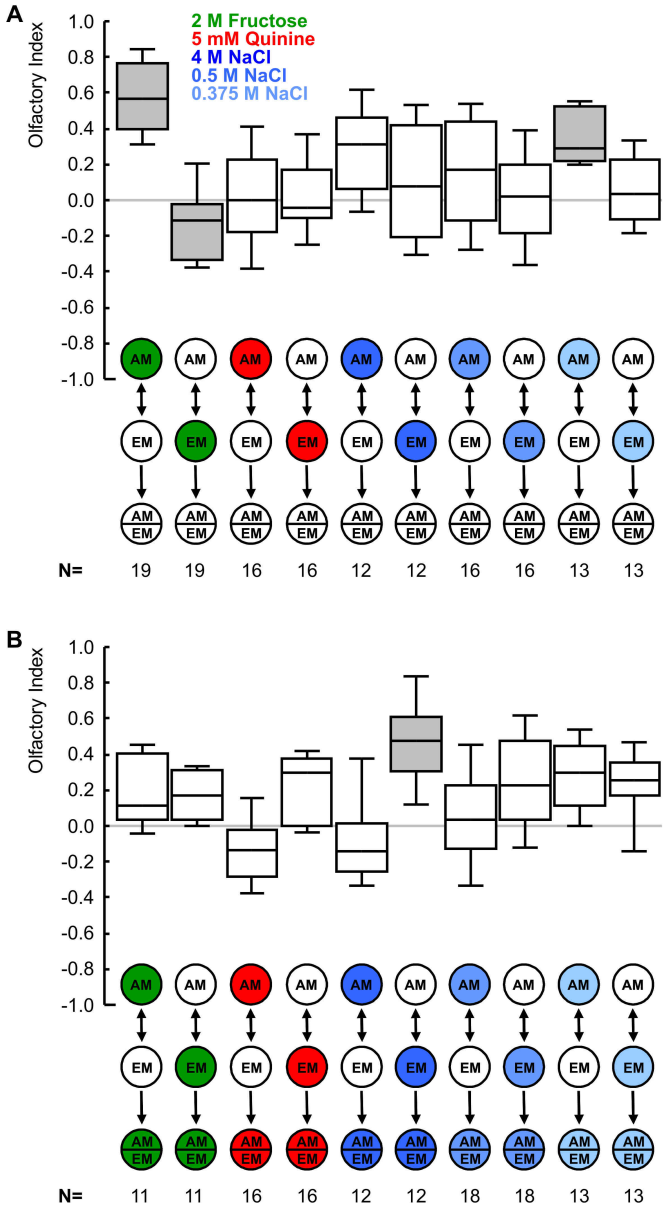


Figure S2

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 5. The sketches below the boxes show the training procedures and test conditions; colored circles represent Petri dishes containing tastant, white circles represent tasteless, pure Petri dishes. Note that differences in OI scores between two corresponding reciprocally trained groups (e.g. the two left-most panels in A) result in Performance Index (PI) scores different from zero (see Fig. 5A, left-most panel). Also note that in half of the cases the sequence of training trials is as indicated (in the left-most panel of (A) e.g. AM+/ OCT), but in the other half is reverse (e.g. OCT/ AM+). For a description of the box plots see legend of Figure 1. Significant differences from zero ($P<$ 0.05/ 10, one-sample sign tests) are indicated by shading of the boxes.

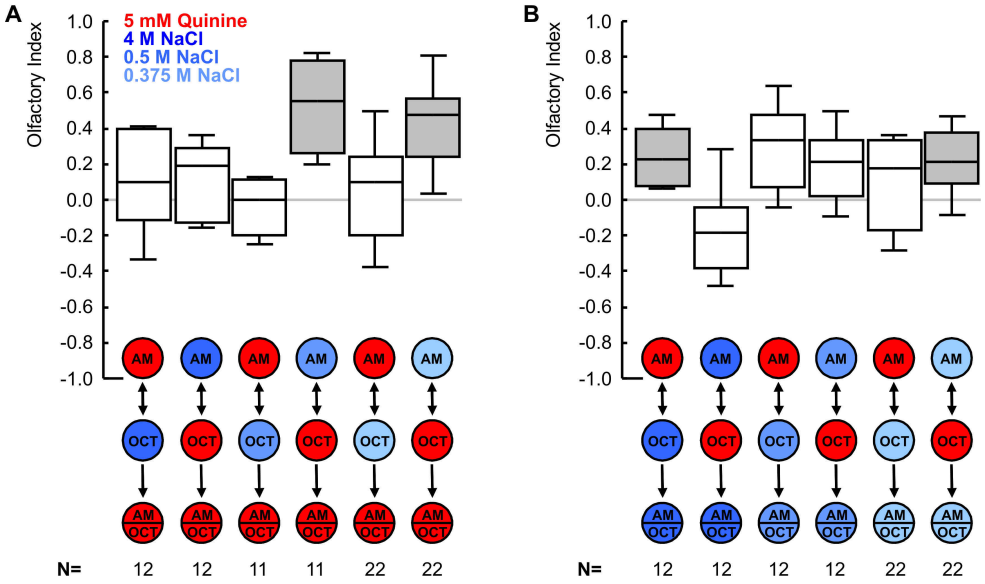


Figure S3
Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 6. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P<$ 0.05/ 6, one-sample sign tests) are indicated by shading of the boxes.

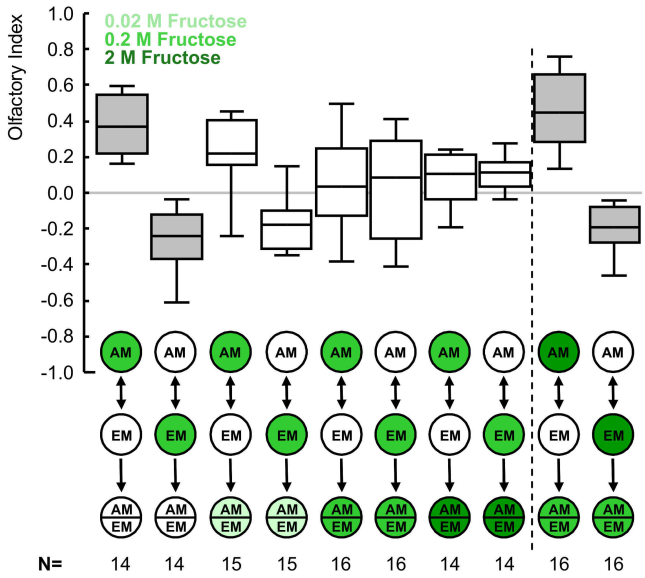
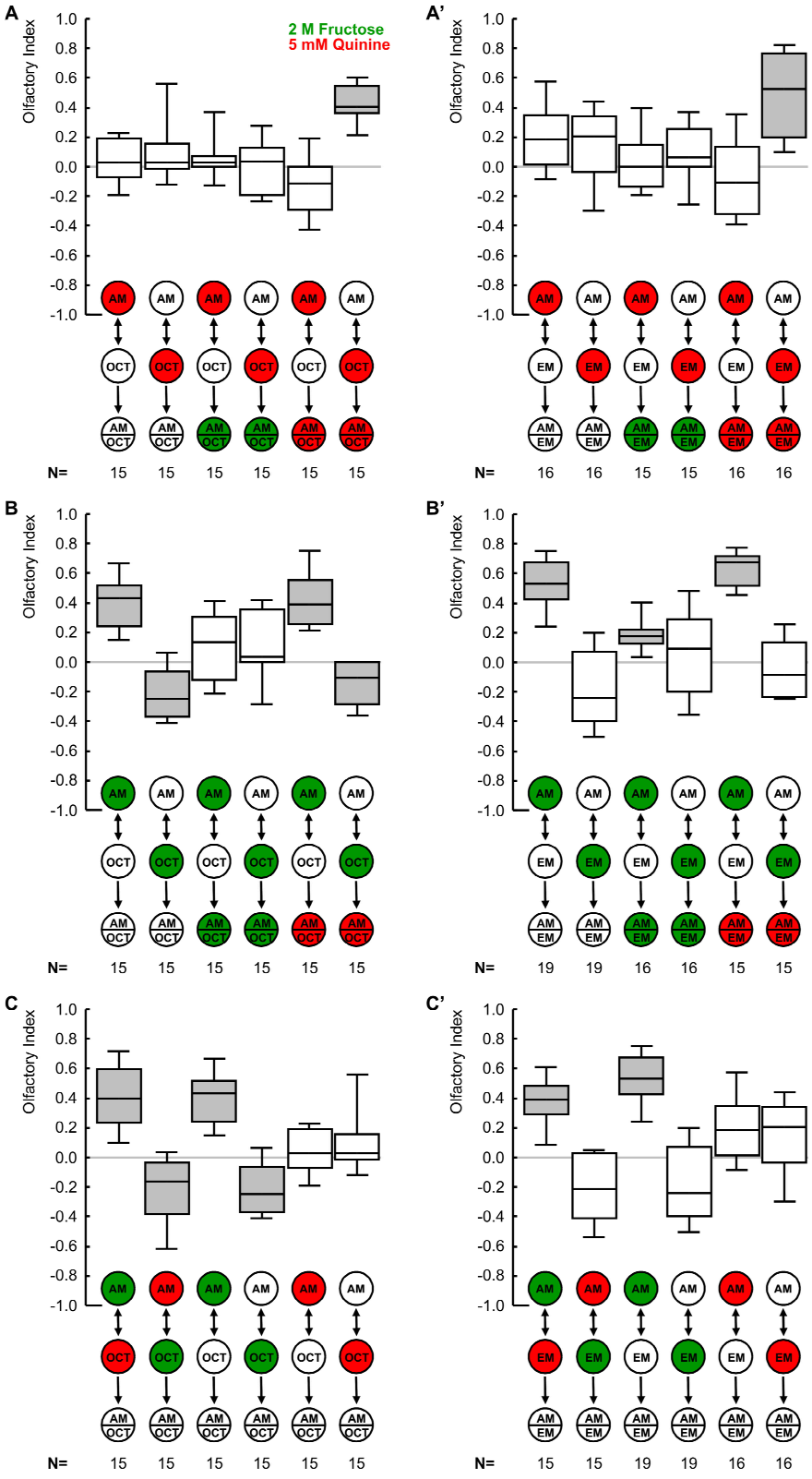


Figure S4
Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 7. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P<$ 0.05/ 10, one-sample sign tests) are indicated by shading of the boxes.


A
A'
B
B'
C
C'
Olfactory Index
2 M Fructose
5 mM Quinine
AM
OCT
EM
N=
15 15 15 15 15 15
16 16 15 15 16 16
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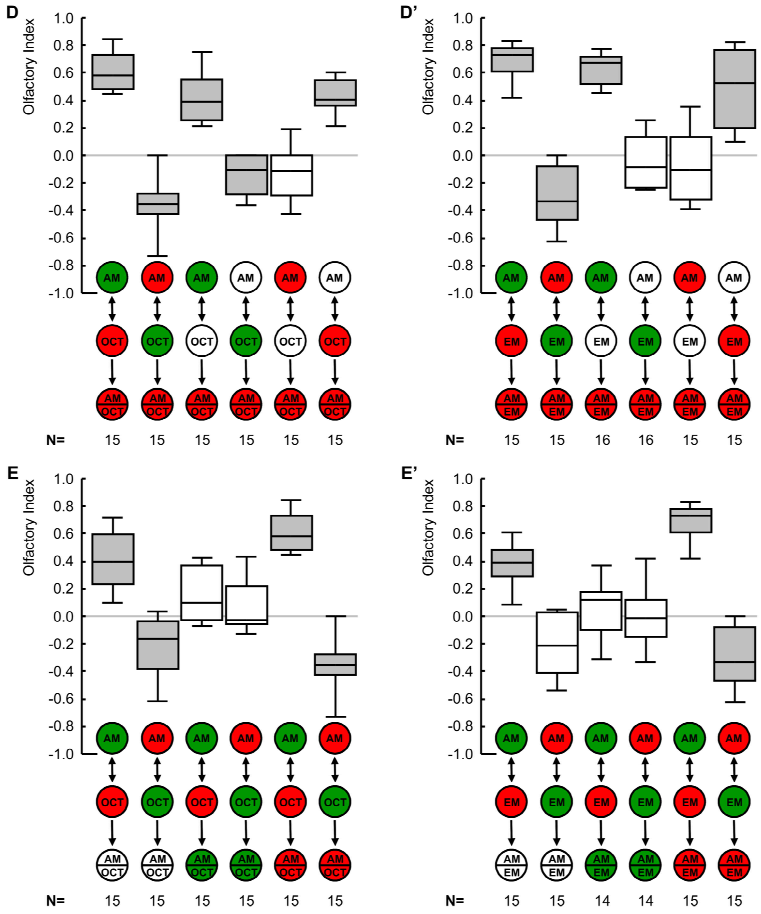

Figure S5
Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 8. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P<$ 0.05/ 6, one-sample sign tests) are indicated by shading of the boxes.

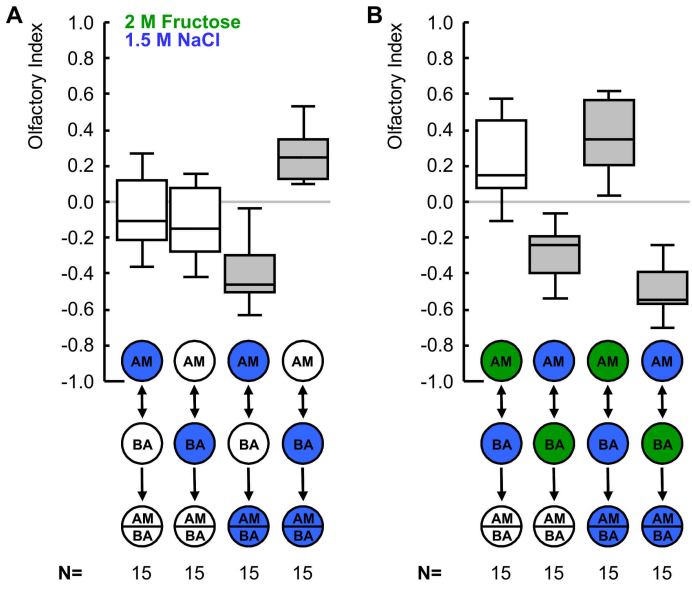

Figure S6
Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 9. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P<$ 0.05/ 4, one-sample sign tests) are indicated by shading of the boxes.

I.2 'Decison-making' in larval *Drosophila*

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Abstract

The brain is the organ of behavior organization. It structures the solution to the problem what to do. This is complicated because usually we cannot be certain which behavior would be the relatively best. These processes, taking place between the moment when an uncertainty between behavioral options is recognized and the actual expression of behavior, are here regarded as 'taking a decision'. Such decision making needs to integrate (i) sensory input, (ii) the current status reflecting evolutionary and individual history, (iii) the available behavioral options, and (iv) their expected outcomes. We specifically focus on the decision to behaviorally express an associative memory trace - or not. After sketching the architecture of the chemobehavioral system in larval *Drosophila* we present a working hypothesis of odor-taste associative memory trace formation, and then discuss whether outcome expectations contribute to the organization of conditioned behavior. We argue that indeed conditioned olfactory behavior is organized according to its expected outcome, namely toward finding reward or escaping punishment, respectively. Conditioned olfactory behaviors thus are not responsive in nature, but rather are actions expressed for the sake of the sought-for reward and the attempted relief. In addition to the organization of such outcome expectations, we discuss parametric features ('axes') of behavioral tasks that we believe bear upon the decision character of the underlying process, and whether these features can be found, or may reasonably be sought for, in larval *Drosophila*. It is argued that rather than trying to draw a line between behavioral processes that reflect decisions versus those that are not, it is more useful to ask *how strong* the decision character of a given behavioral faculty is.

Should I stay or should I go now?
Should I stay or should I go now?
If I go there will be trouble
And if I stay it will be double.
(…)
This indecision's bugging me
Exactly whom I'm supposed to be.
THE CLASH, 1982

Introduction

Decision making is a complex psychological process, and as such the neurosciences alone cannot grasp it in all facets. This is particularly obvious for decision making in animals, where verbal behavior cannot be used for the analysis. Still, insects allow for experimental approaches with enticing analytical power, and a number of study cases for 'decision making' have been reported: the decision to fight or flight in crickets (Rillich et al. 2011); the switch of locusts between solitary and gregarious phases (Ott et al. 2012) as well as their choice between carbohydrate-rich and protein-rich food sources dependent on physiological state (Simpson et al. 1991; for a related follow-up in *Drosophila* see Ribeiro and Dickson 2010); choice behavior during foraging in bees (Chittka et al 2009; Menzel et al. 2011); the resolution of conflict between contradictory cues in adult *Drosophila* (Tang and Guo 2001); the organization of gap-climbing in adult *Drosophila* (Pick and Strauss 2005) and of run-pause-turn behavior in *Drosophila* maggots (Gomez-Marin et al. 2011), as well as the switch between 'gaits' in leeches (Friesen and Kristan 2007; Kristan 2008), included here as insects *honoris causa*. These cases obviously differ in complexity, and indeed the nature of the underlying processes as decisions invites debate. Our approach, however, will be to not try to draw a line between processes that are decisions and those that are not, but to characterize aspects of behavioral tasks that we believe bear upon *how strong* the decision character of the process is. In this context, we argue that the organization of learned behavior in *Drosophila* larvae offers a particularly fruitful study case, holding a balance between sufficiently interesting yet reduced complexity on the one hand, and experimental tractability on the other.

A decision settles the problem what is the relatively best thing to do. We here view 'decision making' as a process to integrate (i) sensory input, (ii) the current status reflecting the evolutionary and individual history of the animal, (iii) the available behavioral options, and (iv) their expected outcomes. Given that in most cases there are no preconfigured solutions for this integration, decision processes need to negotiate ever-new deals between senses, history, behavioral options, and their

expected consequences, in a way that remains behaviorally silent until the decision is actually taken. As a corollary, much of the required processing is offline from immediate sensory-motor loops. Also, because in principle all behavioral options are 'on the table' of which many are mutually exclusive, we may expect a particularly prominent role of behavioral inhibition. Indeed, a key insight of the early behavioral neurosciences, largely based on the contributions of Erich v. Holst, is that sensory processing, rather than triggering motor output, is reconfiguring the inhibitory balance between multiple internally generated behavioral tendencies (Lorenz 1973; Kristan 2008).

Here, we focus on the decision of behaviorally expressing an associative memory trace - or not. To this end, we first sketch the architecture of the chemobehavioral system in larval *Drosophila*; then we present a working hypothesis of memory trace formation regarding the association between odors and taste reinforcement, followed by a discussion of how outcome expectations are implemented to organize conditioned behavior. Finally, we will present our thoughts on how these processes bear on decision making, and which aspects of the psychological richness of decision making in humans are as yet out of scope in our preparation.

(i) Architecture of the chemobehavioral system

Smell and taste serve behavioral organization in different ways. Odors trigger orienting movements and, dependent on the presumed nature of the odor source, organize search or escape behavior, and prepare for suitable action in case tracking is successful, or should escape fail. In a sense, therefore, olfactory behavior is not about odors, but about odor sources, necessarily involving some 'guesswork' about the relation between the odor and its source. In order for these guesses to be well informed, olfactory circuits feature stages with an enormous potential to discriminate odors, and to attach acquired meaning to them. An interesting set of cases is found for pheromones which already are endowed with an evolutionarily determined and largely fixed behavioral meaning; as a corollary, pheromone systems are functionally and often structurally separate from the general olfactory system, and allow for less behavioral flexibility.

In contrast, taste operates 'downstream' of smell, in the sense that it operates upon immediate physical contact with things. Such proximity entails an entanglement with mechanosensation and thus

of the what of taste with the where of touch, and allows for relatively direct and local sensory-motor loops to organize eating and drinking (similar arguments apply for contact chemosensation in other contexts such as predation, defense, social recognition, aggression, and pupariation, as well as courtship, copulation, and oviposition in adults).

The olfactory system of the larva exclusively draws on 21 olfactory sensory neurons organized in seven triplets within the dome of the dorsal organ (Fig. 1A-D, 2) (Heimbeck et al. 1999; Fishilevich et al. 2005; Kreher et al. 2005, 2008). They pass olfactory information ipsilaterally to the larval antennal lobe, each of them targeting one of 21 spherical and anatomically identifiable 'glomerulus' compartments (Python and Stocker 2002; Ramaekers et al. 2005). Each olfactory sensory neuron typically expresses one type of olfactory receptor (OR) molecule of the *Or* gene family together with the obligatory olfactory co-receptor ORCO, coded by the *Orco* gene (CG10609, formerly known as *Or83b* gene and OR83b receptor, respectively) (Fishilevich et al. 2005; Benton et al. 2006; Pellegrino et al 2011; Vosshall and Hansson 2011). The odor response spectra of these ORs, at the chosen concentrations, are diverse yet typically overlapping, ranging from OR94b that responds to a single from the 27 tested odorants, to OR42a and OR85c which each respond to nine odorants out of this panel (Kreher et al. 2005). In turn, most odorants activate more than one OR and thus more than one olfactory sensory neuron. Information about odor quality can thus be coded by the combinatorial activation of ligand-specific subsets of the 21 olfactory sensory neurons (see Laurent et al 2001 for a discussion of temporal-coding aspects of olfaction). Antennal lobe interneurons (Thum et al. 2011) collect from and distribute information to many if not all glomeruli. In analogy to adult flies these connections likely provide inhibitory feedback between glomeruli for gain control across concentrations (Wilson 2008) (we note that, as these neurons sum up olfactory input across the antennal lobe, they may be involved in determining and/or learning about odor intensity). In turn, in adult flies excitatory projections between antennal lobe glomeruli were found to additionally shape the pattern of activity across the antennal lobe (Wilson 2008), and conceivably there are further functions of these anatomically diverse and inter-individually variable neurons (adult: Chou et al. 2010; larva: Thum et al. 2011).

The information from a given antennal lobe glomerulus is conveyed by typically just one projection neuron (Rameakers et al. 2005; but see Marin et al. 2005) to two sites, (i) the lateral horn, and (ii) the mushroom bodies (see Thum et al. 2011 for the description of up to three projection neurons with apparently broader input regions in the antennal lobe). This branched anatomy of projection neurons is characteristic of insects. As compared to the lateral horn pathway which is providing a relatively 'direct' route toward the motor system, the loop via the mushroom bodies can be seen as a 'detour'. The mushroom bodies' olfactory input region, the calyx, is organized into approximately 34 anatomically identifiable and roughly circularly arranged glomeruli (Marin et al. 2005; Ramaekers et al. 2005; Masuda-Nakagawa et al. 2005, 2009). The approximately 21 projection neurons signal typically to one calyx glomerulus each, synapsing onto approximately 600 mature mushroom body neurons (also called Kenyon cells) (Lee et al. 1999; for a different count based on electron microscopy see Technau and Heisenberg 1982). Interestingly, there is a fairly stereotyped relation between the antennal lobe glomerulus in which a projection neuron receives its input, and the mushroom body calyx glomerulus to which it delivers its output, such that it can be individually identified just like the olfactory sensory neurons, antennal lobe as well as calycal glomeruli. We note that 3-4 calyx glomeruli are 'orphaned' in the sense that their inputs are not as yet characterized. In any event, a given Kenyon cell collects input from an apparently random draw of one to three (Ramaekers et al. 2005) or up to six (Masuda-Nakagawa et al. 2005) calyx glomeruli and thus from up to six projection neurons, covering almost a third of the projection neuron coding space. In turn, each projection neuron diverges to 30-180 mushroom body neurons (for details of this approximation see Gerber and Stocker 2007), contributing to up to a third of the mushroom body coding space. If multiglomerular mushroom body neurons need input from more than one calycal glomerulus to fire, this combined convergent-divergent architecture allows for combinatorial coding and a massive expansion of olfactory coding capability. Importantly, the coding space is immediately and drastically reduced in the very next step toward motor output: The Kenyon cells then connect to relatively few (a reasonable guess may be between one to three dozen; Pauls et al. 2010) output neurons that via an unknown number of synaptic steps link to the motor system (Pauls et al. 2010; for the situation in adults: Ito et al. 1998; Tanaka et al. 2008; Sejourne et al. 2011). These mushroom body output neurons

likely receive input from many to all mushroom body cells, thus 'summing up' the activation in their input section of the mushroom body. The mushroom body-to-output neuron synapse thus can be viewed as a 'watershed', in the sense that the combinatorial identity of odor processing is lost at this stage, as a step to transform information about 'Which odor?' into information about 'What to do'. In any event, contemplating the sketched circuitry, it is important that olfactory information reaches the motor systems both via the relatively direct lateral horn route, and via the mushroom body detour. As will be argued below, these two routes organize innate versus learned olfactory behavior.

The gustatory sensory system is comparably much less well understood (reviews: Gerber and Stocker 2007; Cobb et al. 2009; Montell 2009; Tanimura et al. 2009). It comprises approximately 90 gustatory sensory neurons (Colomb et al. 2007a) (all cell numbers are per body side), located in three external taste organs (terminal organ, ventral organ, and the bulge of the dorsal organ) and three internal taste sensilla (ventral, dorsal, and posterior pharyngeal sensilla) (Singh and Singh 1984; Python and Stocker 2002; Gendre et al. 2004). All these cephalic gustatory sensory neurons bypass the brain hemispheres and instead project to the subesophageal ganglion in a way that depends on the receptor gene(s) expressed and their sense organ of origin (Colomb et al. 2007a). Interestingly, projections from the terminal organ and ventral organ neurons remain ipsilateral while projections from the pharyngeal organs can be either ipsi- or bilateral. From the suboesophageal ganglion, taste information is relayed both to modulatory neurons that detour toward the brain and/or the ventral nerve cord (Melcher and Pankratz 2005; Colomb et al. 2007a; Selcho et al. 2009), as well as directly to (pre-)motor neurons presumably in the ventral nerve cord (Fig. 1B), but the details of these connections remain unknown. What is known, however, is that larvae can detect tastants of various human psychophysical categories such as sweet, salty or bitter substances, but may not be able to distinguish e.g. different kinds of sweet (Hendel et al. 2005; Niewalda et al. 2008; Schipanski et al. 2008; see also Thorne et al. 2004; Montell 2009 regarding adults).

On the molecular level, salt likely is detected by Na^+ channels expressed in the terminal organ (Liu et al. 2003), whereas sugar and bitter detection probably is mediated by G-protein coupled receptors of the *Gr* gene family (Clyne et al. 2000; Dahanukar et al. 2001; Thorne et al. 2004; for review see Montell 2009). From the 68 known *Gr* genes in *Drosophila*, 39 have been shown to be

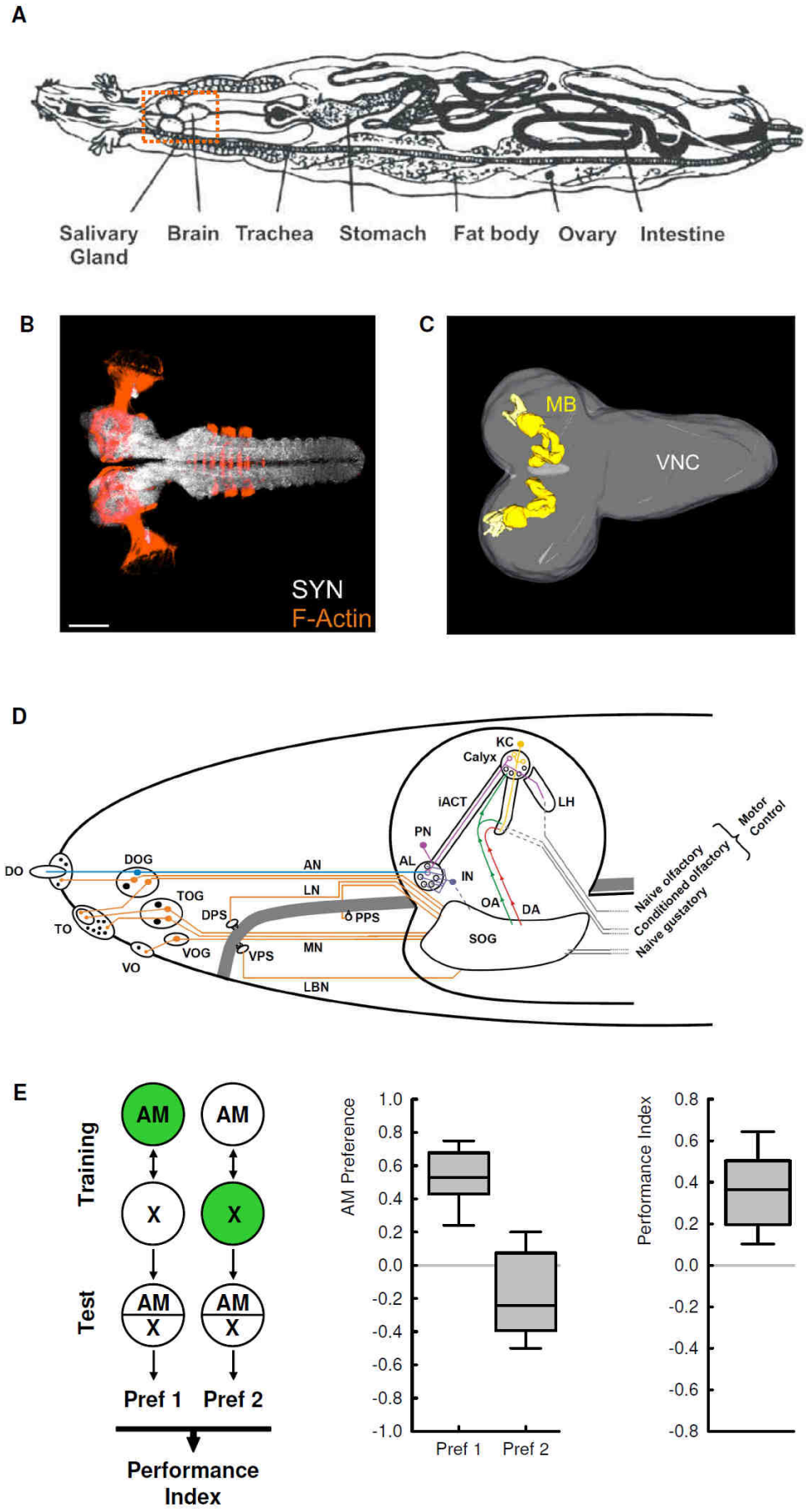

Figure 1. For figure legend see next page.

expressed in the taste organs of the larval head, based on *Gr-Gal4* transgene analysis (Colomb et al. 2007a, Kwon et al. 2011). Strikingly, none of the known adult-expressed sugar-sensitive GRs (*Gr5a*, *Gr64a*, *Gr64f*) (Thorne et al 2004; Dahanukar et al. 2007; Jiao et al. 2007, 2008) is part of these 39 *Gr* genes expressed in the larvae. From the known adult-expressed bitter-sensitive GRs (*Gr33a*, *Gr66a*, *Gr93a*) (Scott et al. 2001; Thorne et al. 2004; Jiao et al. 2007; Lee et al 2009), *Gr33a* and *Gr66a* are expressed also in the larva (Kwon et al. 2011). Lastly, receptors of the *Ir* family (Benton et al. 2009) may participate in gustation in the larval stages.

It is known from adults that contact chemosensory bristles are innervated by two to four gustatory neurons and one mechanosensory neuron, and based on structural (Python and Stocker 2002) and genetic (Awasaki and Kimura 1997) arguments, a similar taste-touch entanglement can be expected in larvae, at least for the external taste organs (discussed in Gerber et al. 2009).

Figure 1 *Basic organization of the larval chemobehavioral system*
(A) Body plan of larval *Drosophila*. The stippled box approximates the area in (B, C).
(B) Immunoreactivity against Synapsin (white) and f-Actin (red) in a 3rd instar larval brain of a wild-type larva. Synapsin immunoreactivity is found throughout the neuropil area of brain and ventral nerve cord. Scale bar: 50 µm.
(C) 3D-Reconstruction of a 3rd instar larval brain. Mushroom-bodies are displayed in yellow, Kenyon cell bodies in pale yellow.
(D) Schematic diagram of the chemosensory pathways in the larval head. Olfactory pathways (blue) project into the brain *proper* and *via* projection neurons (violet) toward the mushroom body (yellow) and the lateral horn, whereas gustatory afferents (orange) are collected in various regions of the suboesophageal ganglion. The green and red arrows indicate pathways to short-circuit a taste-driven reinforcement signal from the suboesophageal ganglion toward the brain.
Abbreviations: AL: antennal lobe; AN: antennal nerve; DA: dopaminergic neurons as participating in reinforcement signalling; DO/ DOG: dorsal organ/ dorsal organ ganglion; DPS: dorsal pharyngeal sensillae; iACT: inner antenno-cerebral tract; IN: antennal lobe neurons (Thum et al. 2011); KC: Kenyon cells; LBN: labial nerve; LH: lateral horn; LI: local interneurons; LN: labral nerve; MN: maxillary nerve; OA: octopaminergic neurons as participating in reinforcement signalling; PN: projection neurons; PPS: posterior pharyngeal sensillae; SOG: suboesophageal ganglion; TO/ TOG: terminal organ/ terminal organ ganglion; VO/ VOG: ventral organ/ganglion; VPS: ventral pharyngeal sensillae.
(E) The larval learning paradigm. A group of 30 larvae is trained such that a sugar reward (green) is paired with *n*-amylacetate (AM) and a pure, tasteless substrate (white) is paired (X) with either a second odor ("two-odor paradigm") or no odor ("one-odor paradigm"). In the subsequent test, the AM preference (Pref 1) is calculated as number of larvae on the AM side minus number of larvae on the other side divided by the total number. A second group of 30 animals is trained reciprocally (Pref 2). The Performance Index as measurement of conditioned behavior is calculated as the difference of Pref 1 and Pref 2, divided by 2. After training with sugar as reward, appetitive memory results in positive Performance Index scores (right-most plot), while negative scores would indicate aversive memory. Please note that in half of the cases the sequence of trials is as indicated, whereas in the other half of the cases the sequence of trials is reverse (not shown). For the aversive learning versions of the paradigm, see body text.
(A) modified from Demerenc and Kaufmann (1972), (B, C) based on data in Michels at al. (2011), (D) based on Stocker (2008), (E) based on data from Schleyer et al. (2011).

(ii) A working hypothesis of memory trace formation

Drosophila larvae not only are able to smell and taste, but also can associate odors with gustatory reinforcement (Fig. 1E) (Scherer et al. 2003, Neuser et al. 2005, Gerber and Hendel 2006, Saumweber et al. 2011; for a manual see Gerber et al. 2010). Such taste reinforcement can either be appetitive (sugar, low-concentration salt) or aversive (high-concentration salt, quinine), such that olfactory preference behavior is respectively increased or decreased. However, from the architecture reviewed so far it appears as if there is no direct connection between olfactory and taste pathways (but see Thum et al. 2011 for the recent anatomical description of neurons innervating both antennal lobe and suboesophageal ganglion). How, then, can odors be associated with tastants?

In 1993, Hammer identified the octopaminergic VUM_{mx1} neuron in the honeybee, which likely receives gustatory input in the subesophageal ganglion and provides output to the antennal lobe, the mushroom body calyx and the lateral horn. This identified, single neuron is activated by unpredicted sugar as well as by sugar-predicting odors, and is sufficient to mediate the rewarding effect of sugar in honeybee olfactory learning (for a description of this neuron in adult *Drosophila* see Busch et al. [2009]; this neuron exists in larval *Drosophila* as well [Selcho et al. 2012]). Also in *Drosophila* there is evidence that octopaminergic neurons can 'short-circuit' taste with smell pathways to mediate appetitive reinforcement signaling (Schwaerzel et al. 2003): Adult flies lacking octopamine because they lack the required enzyme for its synthesis ($T\beta H^{M18}$ allele of the *TβH* gene, CG1543) are impaired in odor-sugar learning but not in odor-shock learning. In turn, blocking synaptic output from a genetically defined set of dopaminergic neurons (*TH*-Gal4) impaired odor-shock learning but not odor-sugar learning. In *Drosophila* larvae, the net effect of driving sets of dopaminergic or octopaminergic/tyraminergic neurons (*TH*-Gal4 or *TDC*-Gal4, respectively) can substitute for punishment or reward, respectively, in olfactory learning (Schroll et al. 2006). However, the genetic tools available at present to manipulate dopaminergic or octopaminergic/tyraminergic neurons cover anatomically and functionally heterogeneous sets of neurons. From these sets of neurons, current research focuses on identifying those conferring appetitive or aversive reinforcement signaling for different memory phases, and to tell them apart from neurons mediating other effects, e.g. regarding olfactory processing, gustatory processing, motor control including the regulation of memory retrieval,

or mediating satiety states (Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009, 2012; for adult *Drosophila*: Claridge-Chang et al. 2009; Krashes et al. 2009; Aso et al. 2010, 2012). Thus, a reasonable working hypothesis appears to be (Fig.s 2,3) that if an odor is presented, a particular pattern of olfactory sensory neurons is activated, dependent on the ligand profiles of the respectively expressed receptors, leading to the activation of a particular combination of glomeruli in the antennal lobe, as well as particular projection neurons and the corresponding lateral horn and mushroom body neurons. At the same time, a tastant, e.g. sugar, activates gustatory sensory neurons that trigger a value signal ('good') *via* e.g. some of the octopaminergic/tyraminergic neurons (in the case of high-concentration salt or quinine a 'bad' signal is delivered *via* e.g. some of the dopaminergic neurons) and send it to many, if not all Kenyon cells of the mushroom bodies. Conceivably, only in that subset of Kenyon cells which are activated coincidently by both the odor signal and the value signal, a memory trace is formed (Tomchik and Davis 2009; Gervasi et al. 2010) (for more detail, see following section). This memory trace is thought to consist of an alteration of the connection between the Kenyon cells and their output neurons: If a learnt odor is subsequently presented, this altered Kenyon cell output is the basis for conditioned behavior. Therefore, the memory trace-forming convergence in the mushroom body is not between the olfactory and the gustatory pathways directly, but between a side branch of the olfactory pathway on the one hand, and a side branch of the gustatory pathway carrying a valuation signal on the other hand.

Regarding the discussion below, two further aspects should be noted: First, the mushroom body loop is largely dispensable, but the projection neurons are required for basic, task-relevant innate olfactory behavior (adult: Heimbeck et al. 2001 and references therein; larvae: Pauls et al. 2010, loc. cit. table S2). This suggests that such innate olfactory behavior is supported by the direct antennal lobe-lateral horn pathway, whereas conditioned olfactory behavior takes the indirect route *via* the mushroom bodies. Second, there is no evidence to argue that a given odor would not activate the same one subset of Kenyon cells during aversive learning as well as during appetitive learning; this implies that appetitive and aversive memory traces for a given odor may be localized in the same Kenyon cells, but in functionally and maybe structurally distinct subcellular compartments, connected to separate output neurons (Fig. 2).

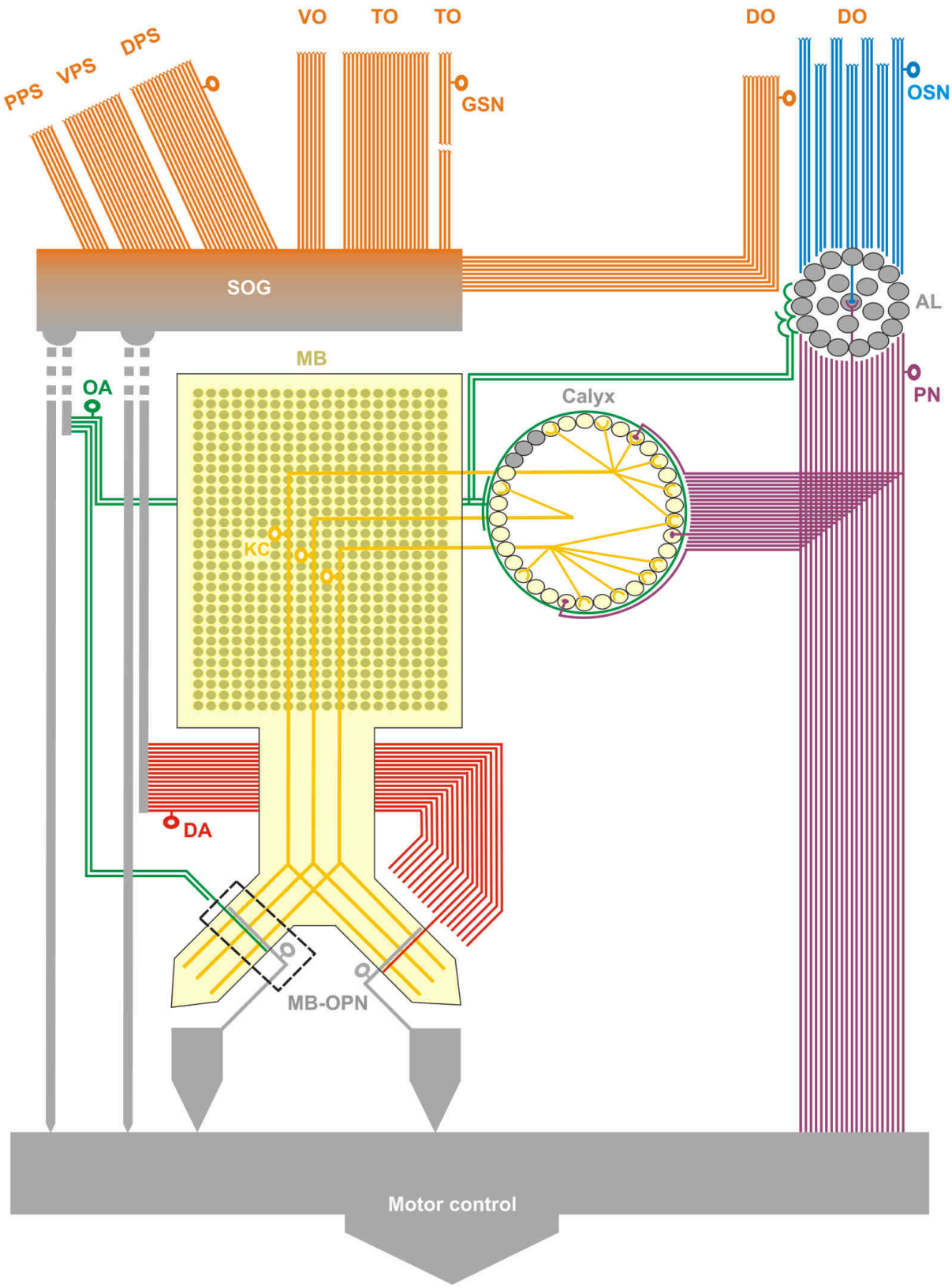

Figure 2. For figure legend see next page.

Figure 2 *Simplified wiring diagram of the larval chemobehavioral system*
Illustration of the numbers and connectivity architecture of neurons of the larval chemobehavioral system as discussed in this review. Please note that (i) for clarity, some neurons (e.g. antennal lobe interneurons: see Fig. 1D or non-Kenyon cell mushroom body intrinsic neurons) are omitted and the topology of the cells, cell bodies, nerves, and brain areas is only partially captured; also for clarity (ii) in many cases only 'example' neurons are drawn in full to illustrate patterns of connectivity for example in the antennal lobe; note that the OA neurons likely communicate toward the calyx and the AL in their entirety; (iii) the polarity of the aminergic neurons is only partially known; (iv) the interruption of the pathway of some GSNs originating in the TO indicates that these neurons, together with the OSNs and the GSNs from the DO, travel via the antennal nerve to the SOG; (v) the grey fill of some calyx glomeruli indicates that their inputs are not classical PNs. Lastly, we would like to draw attention to the lack of knowledge about the connectivity within the SOG as well on the motor side of the system. The area boxed by the stippled line is displayed in Figure 3.
Abbreviations and color code are as in Figure 1D, and in addition: GSN: gustatory sensory neurons; MB-OPN: mushroom body output neuron; OSN: olfactory sensory neurons.

Figure 3 *Working-model of associative memory trace formation*
The leftmost panels (A-D) illustrate the microcircuitry in the mushroom body lobes, the rightmost panels (A'-D') sketch the molecular processes proposed.
(A, A') Before associative training, the presentation of an odor alone activates a particular subset of Kenyon cells (yellow); the amount of transmitter released from that set of Kenyon cells, however, is not sufficient to activate the output neuron(s). Action-potential triggered presynaptic calcium influx is sufficient to allow fusion of synaptic vesicles from the readily releasable pool, but not for a substantial activation of the adenylate cyclase.
(B, B') Before associative training, an aminergic reward signal alone (green) conveyed to most if not all Kenyon cells does not result in activity in the output neurons, and the activation of the G-protein coupled receptor is not sufficient to substantially activate the adenylate cyclase, either.
(C, C') During training, the coincidence of odor-induced activation of the Kenyon cell and of the aminergic reinforcement signal is detected by the adenylate cyclase, such that cAMP is produced, PKA is activated, Synapsin phosphorylated, and reserve-pool vesicles are recruited to the readily releasable pool, where they remain until the Kenyon cell is activated again. That is, during training the Kenyon cells can draw only on the then-existing readily releasable pool, but not yet on the reserve-pool of vesicles to be recruited upon an ensuing reinforcement signal. Thus, the mushroom body output neuron remains silent during training.
(D, D') After training, the now enlarged pool of readily releasable vesicles in the Kenyon cells is sufficient to allow the trained odor to activate the mushroom body output neuron, which is the basis for conditioned behavior.
(E, E') Presenting previously non-trained odors cannot activate the mushroom body output neuron (E: discrimination), unless the set of Kenyon cells activated by them sufficiently overlaps with that set of Kenyon cells representing the trained odor (E': generalization). This discrimination-generalization balance could be adaptively adjusted by e.g. changing the excitability of all Kenyon cells and/or of the mushroom body output neuron.
Color code and abbreviations as in previous Figures, and in addition: AC: adenylate cyclase; ATP: adenosine-tri-phosphate; Ca^{2+}: calcium ion; cAMP: cyclic adenosine-monophosphate; GPCR: G-protein couple receptor; PKA: protein kinase A; RRP: readily release pool; RP: reserve pool; SV: synaptic vesicle; Syn: Synapsin protein.

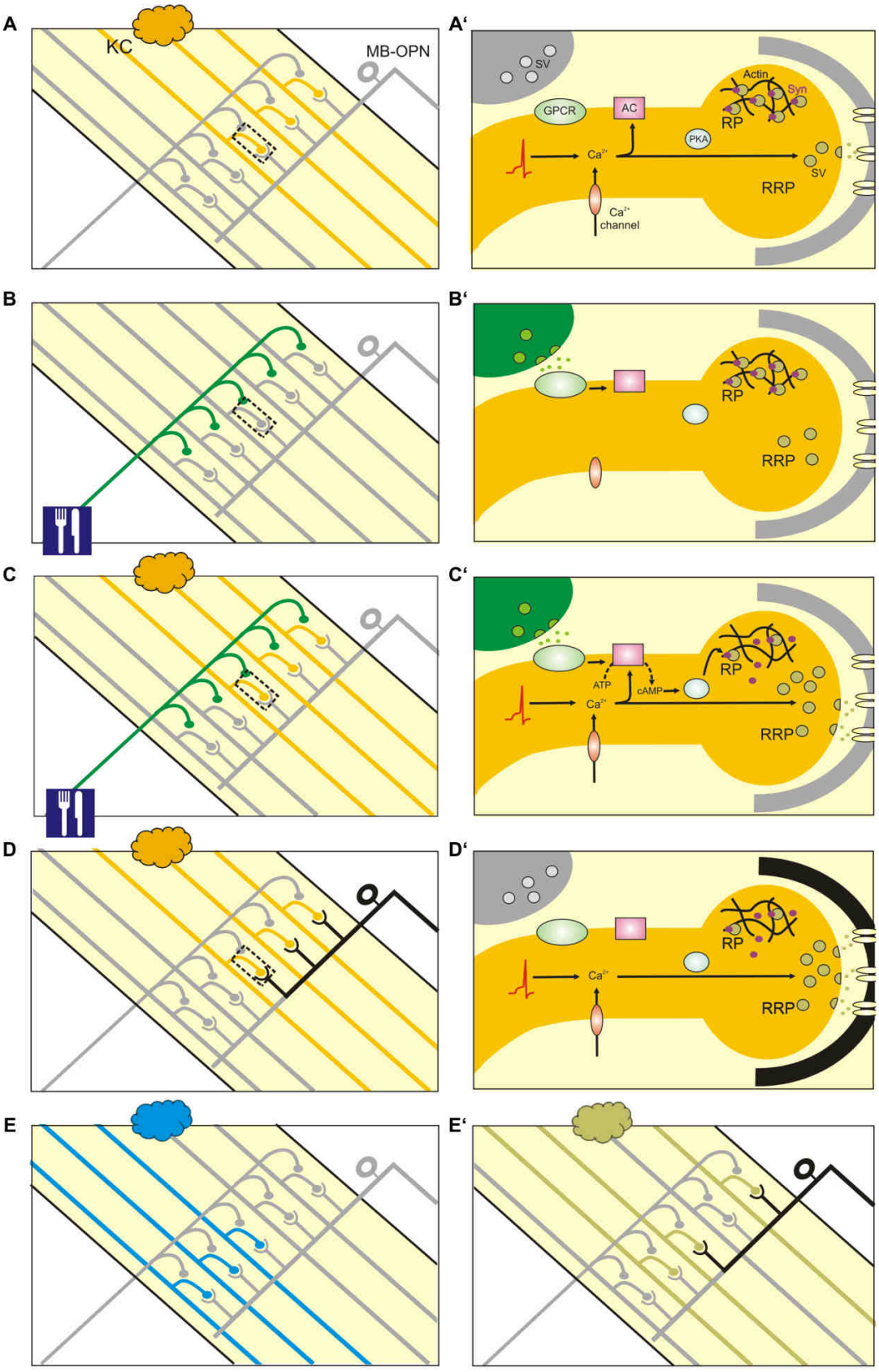

Figure 3. For figure legend see previous page.

Molecular coincidence detection and the AC-cAMP-PKA cascade

As discussed above, a memory trace is formed in those Kenyon cells that are activated by both the odor signal and the value signal – but how is this coincidence detected at the molecular level (Fig. 3)? From research in adult flies (Tomchik and Davis 2009; Gervasi et al. 2010; references therein) it is likely that the *rutabaga* type I adenylyl cyclase (AC) (*rut* gene, CG9533) acts as molecular coincidence detector between the reinforcer and the olfactory activation of the mushroom body neurons: On the one hand, the odor leads to presynaptic calcium influx, and hence to an activation of calmodulin, in that subset of mushroom body neurons that is activated by this odor. On the other hand, the reinforcer activates aminergic neurons and hence the respective G-protein coupled amine receptors in many if not all mushroom body neurons. Critically, it is only by the simultaneous activation of the calmodulin ('odor') and G-protein pathways ('reward' or 'punishment', respectively), that the AC is substantially activated. Thus it is only in those mushroom body neurons which receive both odor and reinforcement activation that cAMP levels and PKA activity are boosted, and the respective protein substrates get phosphorylated. Note that reinforcement signals for reward and punishment apparently innervate different domains of the mushroom bodies (Pauls et al. 2010).

While this working hypothesis of memory trace formation seems to reasonably integrate most of the available data in adults as well as in larvae, the actual effector proteins that are phosphorylated by PKA to support fly short-term memory remained uncertain (for *Aplysia* see Hawkins 1984). To this end, we ventured into an analysis of Synapsin function (Michels et al. 2005, 2011).

Memory trace and Synapsin function

Synapsin is an evolutionarily conserved phosphoprotein associated with synaptic vesicles (for a review of *Drosophila* Synapsin: Diegelmann et al. in press; regarding other study cases: Benfenati [ed.] 2011). In flies Synapsin is coded by a single gene (*syn*, CG3985) (Klagges et al. 1996). It is dispensable for basic synaptic transmission (Godenschwege et al. 2004), and can bind to both synaptic vesicles and cytoskeletal actin, forming a so-called reserve pool of vesicles. Importantly, phosphorylation of Synapsin allows synaptic vesicles to dissociate from this reserve pool and become

part of the readily releasable pool of synaptic vesicles such that they are eligible for release upon a future action potential.

On the behavioral level, larvae carrying the protein-null deletion syn^{97} suffer from a 50 % reduction of associative function in the odor-sugar learning paradigm (Michels et al. 2005) (adult odor-shock learning is likewise impaired: Godenschwege et al. 2004; Knapek et al. 2010); this defect can be phenocopied by transgenic down regulation of Synapsin using RNAi (Michels et al. 2011). The syn^{97} mutant shows intact abilities to recognize gustatory and olfactory stimuli and the respectively needed motor faculties, and is not differentially sensitive to experimental stress, sensory adaptation, habituation, or satiation as compared to its wild type genetic background (Michels et al. 2005). Using a series of rescue experiments by expressing transgenic Synapsin in various parts of the larval brain by means of a UAS-*syn*cDNA effector construct, the follow-up study of Michels et al. (2011) analyzed where in the larval brain a Synapsin-dependent memory trace is localized. After showing that an acute rescue is possible (*elav*-Gal4 in combination with *tub*-$Gal80^{ts}$), it turned out that Synapsin expression in the mushroom body (*mb247*-Gal4 or *D52H*-Gal4) is sufficient to fully rescue the syn^{97}- mutant defect in associative function (Fig. 4). Expression of Synapsin in projection neurons (*GH146*-Gal4 or *NP225*-Gal4) is not sufficient for a rescue; notably, this lack-of-rescue cannot be attributed to adverse effects of the driver constructs used. Furthermore, restoring Synapsin in fairly wide areas of the brain but not in the mushroom bodies (*elav*-Gal4 combined with *mb247*-Gal80) is not sufficient for rescuing the learning defect of the syn^{97} mutants, and also in this case adverse effects of the transgenes cannot be held responsible for such lack-of-rescue. Thus, it appears that a Synapsin-dependent short-term memory trace is localized to the mushroom bodies, and the mushroom bodies may turn out to be the only site for such a memory trace.

At the molecular level, it was argued above that the associative coincidence of odor and appetitive reinforcement boosts AC-cAMP-PKA signalling. Interestingly, *Drosophila* Synapsin contains a number of predicted phosphorylation sites, including the evolutionarily conserved PKA/CamK I/IV consensus motif RRFS at Ser^{6}, an evolutionarily non-conserved PKA/CamK I/IV consensus motif RRDS at Ser^{533}, and suspected sites for other kinases, such as CamK II, prolin-dependent kinase, and PKC (Nuwal et al. 2011). Therefore, Michels et al (2011) tested whether a

Synapsin protein with the two PKA/ CamK I/IV sites mutationally disabled (UAS-*syn*cDNA-PKA$^{S6A/S533A}$) can rescue the associative defect of the *syn*^{97} mutants. As no such rescue was observed, it seems likely that Synapsin exerts a function in memory trace formation as a substrate of the AC-cAMP-PKA cascade.

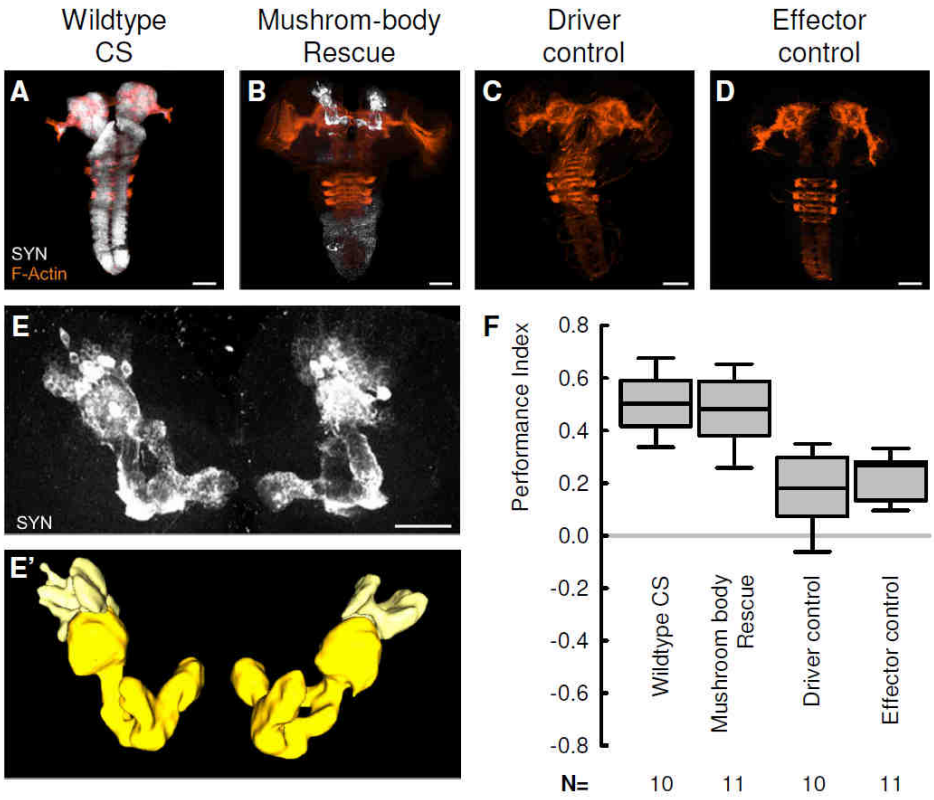

Figure 4 *A local Synapsin-dependent memory trace in the mushroom bodies*
(A-D) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. (E) A magnified view of the mushroom bodies from the mushroom-body rescue strain, using *MB247*-Gal4 as driver; (E') 3D reconstruction of the mushroom bodies. (F) Using the two-odor paradigm, associative function is impaired in both driver and effector control (because these are in the *syn*^{97}-mutant background) and is fully rescued in the mushroom-body rescue strain (which certainly also is in the *syn*^{97}-mutant background). Scale bars: 50µm in A-D, 25µm in E. The same full rescue is observed upon acute Synapsin expression, as well as for another mushroom-expressing driver strain. No rescue is observed for drivers expressing in projection neurons, or when expressing Synapsin in wide areas of the brain yet excluding the mushroom bodies (see body text).
From Michels et al. (2011).

(iii, iv) The decision to behaviorally express a memory trace – or not

The above discussion suggests that the formation of an odor-sugar associative memory trace involves an interaction between the mushroom body side branch of olfactory processing and a modulatory reward signal branching off of the taste pathway. The experiments reviewed in the following (Gerber and Hendel 2006; Eschbach et al. 2011; Schleyer et al. 2011) investigate how the process of 'translating' such a memory trace into conditioned behavior is organized.

When larvae are trained with a medium concentration of fructose, they do not automatically show conditioned behavior during the test (four leftmost plots in Fig. 5A): If tested in the presence of various fructose concentrations, larvae show conditioned behavior when the sugar concentration at the moment of test is lower than the sugar concentration during training, whereas animals tested on a substrate with a sugar concentration equal to or higher than during training do not. These differences in conditioned behavior are puzzling, because the equal training with the medium sugar concentration induces the same memory trace in all these groups. One interpretation of this result could be that medium or higher sugar concentrations during the test altogether prevent conditioned behavior. This is not the case, however: Conditioned behavior actually *is* possible in the presence of a medium sugar concentration, provided the training-concentration was yet higher (rightmost plot in Fig. 5A)! Thus, neither the training concentration *per se* nor the testing concentration of sugar *per se* determines conditioned behavior - but their comparison does.

Our interpretation is that conditioned behavior toward food-associated odors is a *search for food*, which is abolished in the presence of the sought-for food. This is a fundamental difference in perspective to regarding conditioned behavior as *response to the odor*. The animals apparently compare the value of the activated memory trace with the value of the testing situation and show appetitive conditioned behavior depending on the result of this comparison. That is, conditioned search is enabled only if the outcome of tracking down the learned odor promises a gain in the sense of yet-more-reward than is actually present:

Conditioned search if: Appetitive Memory > Observed Reward

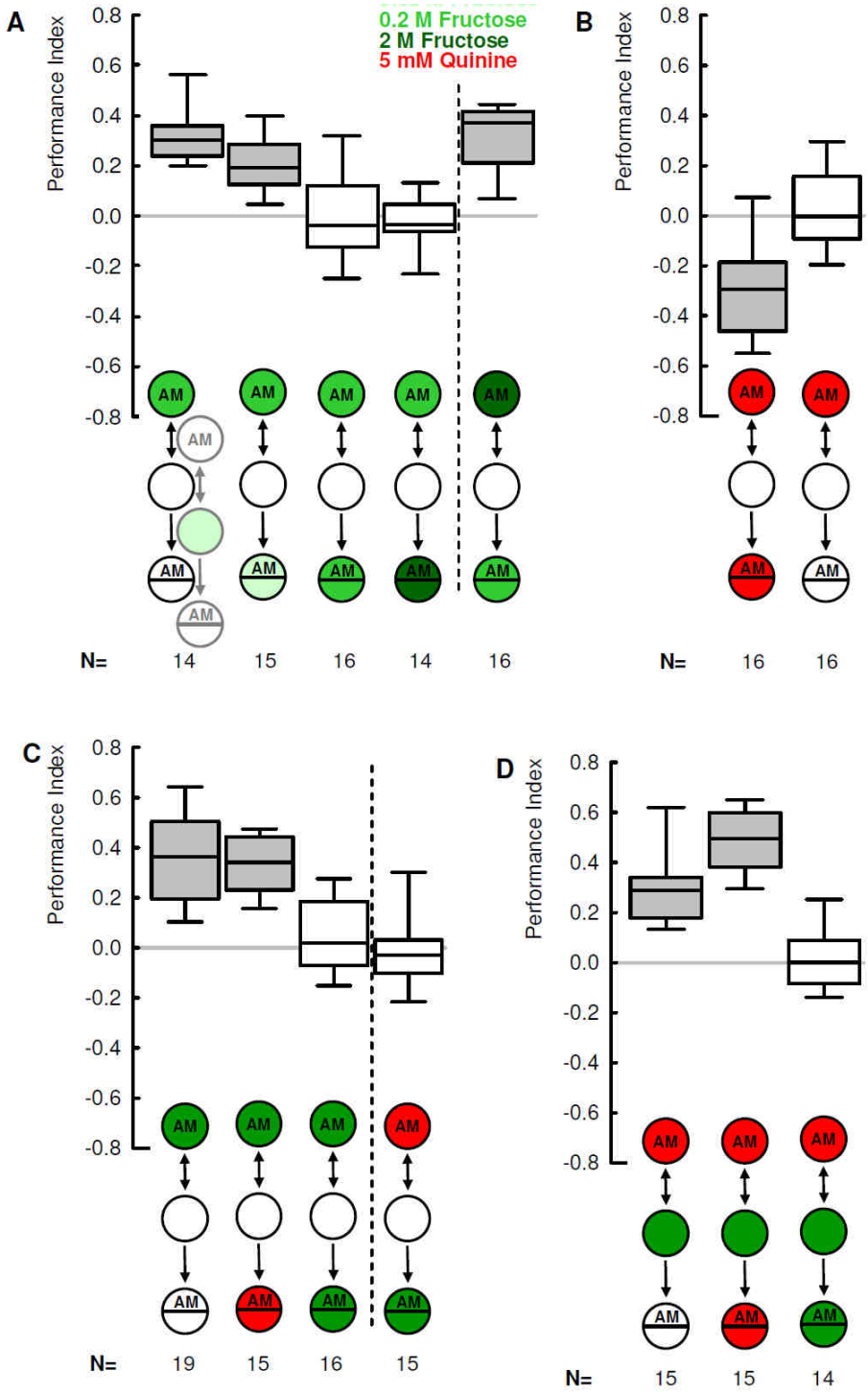

Figure 5 *Expected gain drives learned olfactory behavior*
(A) Animals are trained using n-amylacetate (AM) and empty odor cups (EM). In all four left-most panels a medium fructose concentration (0.2 M) is used as reinforcer during training; the subsequent test is performed either in absence of fructose, or in presence of a lower-than-trained fructose concentration (0.02 M), the training-concentration of fructose (0.2 M), or a higher-than-trained fructose concentration (2 M). In the right-most panel, a high fructose concentration (2 M) is used during training, but the test is performed in the presence of the medium (0.2 M) fructose concentration. Memory is behaviorally expressed only if the fructose concentration during training is higher than the fructose concentration at the moment of test.
(B) After aversive-only training, larvae behaviorally express memory only in the presence of quinine.
(C) In contrast, after appetitive-only training (three left-most panels), memory is behaviorally expressed only in absence of fructose, whereas the presence of quinine has no effect. Conversely, after quinine-only training the presence of fructose has no effect (right-most plot).

Figure 5 continued
(D) After push-pull training, scores for animals tested on quinine are higher than for those tested on pure, suggesting that only under these conditions both appetitive and aversive memories are behaviorally expressed.
Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero (one-sample sign tests, [A] $P< 0.05/ 5$, [B] $P< 0.05/ 2$, [C] $P< 0.05/ 4$, [D] $P< 0.05/ 3$) are indicated by shading of the boxes.
Based on data from Schleyer et al. (2011).

This comparison clearly requires the memory trace to be read out. Thus on the cellular level the point of comparison between the memory trace and the reward present during testing must be downstream of the memory trace, and according to the above working hypothesis of memory trace formation downstream of mushroom body output. This is therefore a process different from the effect of satiety which as suggested by Krashes et al. (2009) acts on the α/β lobes of the mushroom body (Krashes et al 2009, loc. cit. Fig. 7) to altogether silence mushroom body output at test.

What, then, occurs during conditioned behavior after aversive conditioning? Associative avoidance scores after quinine training are revealed in the presence but not in the absence of quinine (Fig. 5B). Such behavior can be understood if we regard behavior after aversive conditioning as *escape from quinine*, which is pointless in the absence of a reason for an escape (the same was found for high-salt [Gerber and Hendel, 2006; Schleyer et al. 2011] and mechanical disturbance [Eschbach et al. 2011] as punishment). Thus, conditioned escape behavior remains disabled unless such conditioned escape offers a gain in the sense of relief from punishment:

No conditioned escape if: Aversive Memory > Observed Punishment

Whether there is the same kind of quantitative comparison between memory trace and testing situation as in the appetitive case (see above), however, remains to be directly shown for aversive memories. Based on experiments using quinine and differently high concentrations of salt, this seems likely, though (see discussion in Schleyer et al. 2011).

Interestingly, the behavioral expression of a fructose-memory trace is independent of the presence of quinine (Fig. 5C; two left-most groups), and likewise the presence of fructose does not prompt conditioned escape after quinine training (Fig. 5C; right-most group). Thus, the modulation of

conditioned behavior by the gustatory testing environment can be exerted independently onto appetitive and aversive conditioned behavior. Once these respective modulations do take place, though, the resulting behavioral tendencies can certainly summate: After associating one odor with fructose and the other odor with quinine, scores from a choice situation between these two odors are higher in the quinine testing condition than in the pure testing condition (Fig. 5D). This, as we argue, is because in the presence of quinine both memory traces can be expressed behaviorally: Conditioned escape from quinine is expressed because *quinine is present*, and conditioned search for fructose is expressed because *fructose is absent*.

Thus, conditioned olfactory behavior is organized in a flexible way, according to its expected outcome, namely toward finding reward and escaping punishment. Conditioned olfactory behavior therefore is not responsive in nature, but rather is an action expressed for the sake of those things that are not (yet) there, the sought-for reward and the attempted relief.

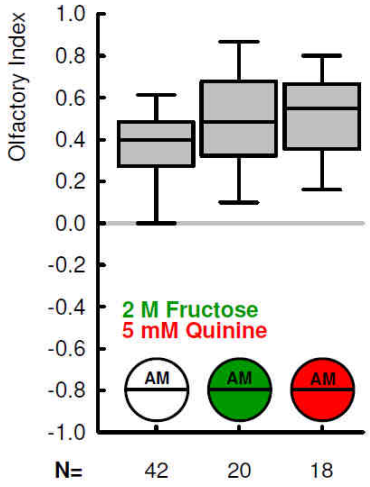

Figure 6 *Innate olfactory behavior is unaltered in the presence of tastants*
The Olfactory Index for n-amylacetate diluted 1:50 in paraffin oil does not differ on the indicated substrates. Pooled data are significantly different from zero ($P < 0.05$, one-sample sign test).
Based on data from Schleyer et al. (2011).

Independence of innate olfactory and innate gustatory behavior

Technically speaking, the experiments detailed above imply that taste processing affects conditioned olfactory behavior. This raises the question whether the same is true for innate (in the sense of experimentally naïve) olfactory behavior. This is not the case: Larvae show indistinguishable innate olfactory choice behavior on various tastant substrates (Fig. 6), even when odors are diluted to yield

only moderate levels of attraction which arguably are easier to be modulated (Schleyer et al. 2011). In turn, innate gustatory behavior is not influenced by odor processing, either (Schleyer et al 2011).

Thus, innate olfactory and innate gustatory behavior seem to be mutually 'insulated' from each other, and in this sense seem to be responsive in nature.

A circuit for decision making?

We are thus confronted with two different kinds of olfactory behavior: Innate, largely hard-wired and responsive olfactory behavior, and flexibly organized learned olfactory behavior which is better captured as an action in pursuit of its outcome. Considering the functional circuitry of the larval chemobehavioral system, these two kinds of olfactory processing can be mapped onto the direct antennal lobe-lateral horn pathway on the one hand, and the mushroom body loop on the other (Fig. 7). Regarding the topic of this contribution, namely decision making, we would like to draw attention to the site of the chemobehavioral system labeled by (4a,b) in Figure 7. At the moment of testing, an integration takes place at this site between the four aspects of decision making introduced above: (i) sensory input in terms of the gustatory interneuron reporting on the value of the current situation, (ii) the animal's history in terms of the activated memory trace from the mushroom body, (iii) the available behavioral options in terms of the connections to motor control, and (iv) the predicted consequences of executing these options in terms of the calculated difference between (i) and (ii). We therefore argue that the process of behaviorally expressing an odor-taste memory trace, or not doing so, features fundamental properties of decision making. We believe that this process is simple enough that it can eventually be understood at satisfying cellular and molecular detail, yet is complex enough to warrant experimental effort. Still, this process seems to lack much of the psychological complexity of decision making as we experience it ourselves in our lives. What are the issues?

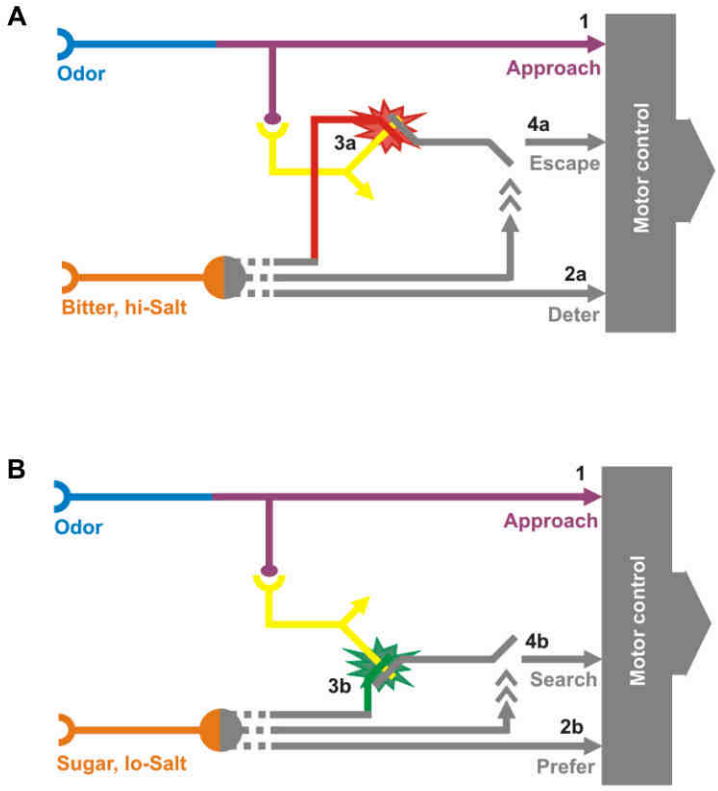

Figure 7 *A minimal, behavior-based circuit for larval decision making*
Based on the neuroanatomy presented in Figure 2 and the behavioral findings presented in Figures 5 and 6, we propose a behavior-based circuit of chemosensory behavior and decision making in larval *Drosophila* regarding the decision to behaviorally express an appetitive or an aversive (B) memory trace- or not.
(A) (1) Odors innately are usually attractive (Fig. 6) (for exceptions see Cobb and Domain 2000; Colomb et al. 2007b). (2a) Larvae innately are deterred by quinine and high concentrations of salt (Hendel et al. 2005; Niewalda et al. 2008) and (3a) can associate an odor with such punishment, leading to conditioned aversion to this odor (Fig. 5B; Gerber and Hendel 2006; Niewalda et al. 2008). This requires convergence and coincidence detection of olfactory and aversive reinforcement processing (red star symbol). (4a) Olfactory memory traces are behaviorally expressed only if animals expect to improve their situation: only if at the moment of testing a punishment signal is present that is at least as 'bad' as predicted, conditioned escape behavior is enabled (Fig. 5B).
(B) (2b) Larvae innately prefer sugar and low concentrated salt (Schipanski et al. 2008; Niewalda et al. 2008), and (3b) can associate an odor with such reward, leading to conditioned approach toward this odor (Fig. 5A; Scherer et al. 2003; Neuser et al. 2005; Schipanski et al. 2008), requiring convergence and coincidence detection of olfactory and appetitive reinforcement processing (green star symbol). (4b) The presence of a reward signal at the moment of the test that is at least as 'good' as predicted disables the expression of conditioned search behavior (Fig. 5A).
Based on Schleyer et al. (2011).

(v) Aspects of decision making

As we have argued, the brain is the organ of behavior organization. At any moment, it structures the solution to maybe the most 'bestial' of all problems: Should I stay or should I go? To this end, the brain integrates (i) sensory input, (ii) the subject's individual and evolutionary history as reflected in its internal status, (iii) the available behavioral options, and (iv) their expected consequences. As a corollary of this integrative function, many events in the brain are related to various kinds of processing offline sensory-motor loops. Such offline processing is required because it is usually not clear which behavior will be best: Behaviors that are expressed always if and only if there is a particular input, i.e. behaviors that are expressed largely irrespective of other situational or internal conditions, are rare exceptions, in the case of monosynaptic reflexes, or appear pathological in the case

of unbreakable habits. Normally, behavior needs to be acutely tailored to suit the configuration of senses, status, options, and expected consequences. These processes, taking place between the moment when uncertainty between behavioral options is registered (the moment when I ask myself whether I should stay or go) until the eventual expression of behavior (me finally staying or going) we experience as us 'taking a decision'.

Obviously, the task at hand when using such a concept of decision making as 'experiential corollary of behavior organization under uncertainty' is to develop behavioral paradigms that confer an experimental handle on such processes. Furthermore it is obvious that the boundaries of such a concept are fleeting, in particular toward guessing when uncertainty is very high and toward reflexive or habitual behavior when uncertainty is very low. Therefore, we try to 'parametrically' characterize decision making: We characterize five aspects of behavioral tasks which as we suggest bear upon *how strong* the decision character of the process is. These characterizations, based on what we as humans experience when confronted with decisions, will then each be followed by a discussion as to whether these characteristics can be found or can reasonably be sought for in larval *Drosophila*.

Before starting this discussion we note that along sensory-motor loops sensory signals, graded in nature, often need to be dichotomized to command behavior- or not. This process to funnel sensory representations into behavioral categories entails a dilemma: In some situations, it may be warranted to regard recognizably different representations as the same (generalization), while under other circumstances it may be appropriate to regard them as different despite recognizable commonalities (discrimination). Clearly, these kinds of process are fundamental for perception and behavioral organization in any system, and seem experimentally accessible in *Drosophila* (Eschbach et al. 2011b; Niewalda et al. 2011), including the adaptive adjustment of the generalization-discrimination balance in larval *Drosophila* (Mishra et al 2010).

Options

To take a decision, there have to be options. In the simplest case, the options in the face of a particular situation are to express a given behavior- or not. Only highly specialized monosynaptic reflexes, e.g. in the context of escape or postural control, may hardly offer at least this one degree of freedom. As

more options come into play, the process of behavioral organization increasingly assumes the nature of a decision. Clearly, the more strongly the network is biased toward a given behavior, either because of the way it is wired, or because the state of the subject confers an imminent imperative, for example after starvation, the less room there is to take a decision. Similarly, if the differences between the predicted consequences of each behavior are too drastic, the process loses its decision character as well, because uncertainty is lost. In turn, the decision character would get lost, too, if the difference in consequences were too small, such that the subject would have to guess.

In the case of *Drosophila* larvae facing the test situation, they may track down the odor, turn their back on the odor, or ignore it. In the latter case, they may orient with respect to other, inadvertently applied stimuli and/or their conspecifics, start feeding on or digging into the substrate, or pupate. This behavioral richness is overlooked when our assay is performed, as typically only olfactory behavior with respect to the trained odor is scored. For this scored learned olfactory behavior, we have argued that the larvae have the option to behaviorally express it- or not, and that this process is organized at the site of the circuit labeled by (4a,b) in Figure 7.

Dimensions & Conflict

As the number of dimensions in which two behavioral options differ is increased, the process turns into a decision: All else being equal, you right away go for the job with the higher salary. Similarly, all else being equal you go for the job that allows for a greater independence. It is only when the two jobs differ in both dimensions and if there is a conflict that a decision is called for, e.g. if the better-paid job offers less independence. If such a conflict is recognized, you may scale the difference in income between the jobs and the difference in terms of independence according to their priority; if the differential between the jobs remains too small even after such inner bargaining, you may seek for a third dimension, such as the duration of the contract, to tip the balance. The more dimensions are consulted and the more of these iterations take place, the more the process assumes the nature of a decision.

Regarding the test situation in our assay, we have argued that innate and learned olfactory behavior are organized rather separately. Thus, if the innate and the learned value of an odor are

different or even opposite, the larvae may need to prioritize. Likewise, during extinction the larvae may form an extinction memory, namely that a contingency of odor and reward is no longer valid; it would then need to pit such extinction memory against the 'original' memory that does suggest such a contingency. Similarly, if odor-food memories were specific not only for the quality of the trained odor (Chen et al. 2011), but also for its intensity (regarding adults, see Yarali et al. 2009), the organization of test behavior along an odor gradient would be a more complicated matter than it appears to be.

Lastly, we note that the two kinds of logical errors one can make, i.e. false-positive behavior (tracking down an odor although no reward will be available) and false-negative behavior (not bothering to search for reward although it would be available at the odor source), represent a fundamental dimensional conflict for larval as well as any sort of decision making. The way this conflict is negotiated is conceivably different for rewards versus punishments (and in both cases is likely subject to state-dependent modulation), and may interact with negotiating the generalization-discrimination balance mentioned above. Indeed, an adaptive adjustment of the generalization-discrimination balance (Mishra et al. 2011) could be achieved via error-dependent changes in the excitability of mushroom body neurons and/or of mushroom body output neurons.

Time & Certainty

Taking decisions takes time: All else being equal, a given behavior loses its credentials as a decision, rather than a guess, as the time allowed to go through the process is shortened. We note that some flavor of guessing will always remain in behavioral organization, however, because (i) the subject always has to close the process after finite integration time, (ii) predicting the consequences of behavior cannot be fully accurate, and (iii) perceptual hypotheses generated from sensory signals also are error prone. In turn, if for the sake of the argument we assume that all perceptual and predictive processes are perfectly accurate, and time is not limiting, behavior organization would always converge onto that one optimal solution; there would neither be the initial moment of uncertainty as to what to do which is characteristic of the decision process, nor would there be any realistic alternative. Thus, it seems that intermediate levels of perceptual and predictive accuracy as well as intermediate

time budgets are characteristic of decision processes: If accuracy is too low or time too short, one cannot decide - but if accuracy is too high and time were endless there would be no need to decide.

Regarding time, the 3 min allowed for the larvae to distribute seems enough to 'make up their minds', as scores do not get better if more decision time is allowed (Scherer et al 2003). Regarding certainty, one may fancy an experiment where the larvae can opt-out from testing by means of a 'DON'T KNOW' option. As a more sober thought, we note that larvae need more than one odor-reward trial to eventually support conditioned behavior (Neuser et al. (2005), as is the case for most other classical conditioning paradigms, and likewise associative scores decrease as odor or reward intensities decrease. All these three parameters may impact how certain the larvae are about the relation between odor and reward, so one may independently vary these parameters for two odors to pit them against each other. Along the same lines, it may be possible to introduce variance into the odor-reward relationship. If it turns out that odor-reward memories indeed contain information about these parameters of reliability, it would be a challenge to understand which and how such 'meta-data' are encoded.

As a complication, assays based on the behavior of cohorts of animals, as in our case, or on statistics integrating individual animal behavior into one statistic, are ambiguous: A partial associative score may imply that all animals are partially certain, or it may imply that some animals are certain of the contingency between odor and reward while other animals are certain that there is no such relation or are agnostic (for a study of this problem in bees see Pamir et al. 2011). Similar questions may be raised on the synaptic level: Do partial scores come about by all relevant synapses being partially modified, or by fractions of synapses that are while the rest are not?

Pride, Blame & Person

Once a decision and corresponding action is taken, we take note of the consequences. The clearer the decision character of the process was, the more 'personally' we take the consequences, the more readily we take pride in the success, or blame ourselves for the failure. This is different in cases when our behavior had been reflexive or in another way forced, or in cases when we have guessed and then can merely recognize our good or bad luck. Still, both after a successful decision as well as after a

'lucky punch', we are inclined to repeat the behavior under similar circumstances: If after the first guess we are once more lucky with a similar second guess, we will begin to believe in an action-outcome relation, and will incorporate this now 'educated guess' into a future decision process. If such an initial decision indeed yields the intended consequence, we can in the future take less effort in the process, such that eventually the nagging feeling of indecision gets lost. Rather, our behavior will eventually become habitual, forming a reliable aspect of us as persons (see introductory quote).

We believe that the closest behavioral studies can get to a thus-understood 'personal' level is to focus on operant behavior and operant conditioning: A prerequisite for an animal to learn that its actions have particular consequences is that it recognizes itself as 'subject', as author of its actions and decisions. It should be interesting to see whether *Drosophila* larvae, similar to adults (review: Brembs 2009) have such faculties.

Offline processing

As a last, and indeed fundamental aspect, we note that at the beginning of the decision making process, more than one but not all behaviors are practically possible: In a fight-or-flight situation, for example, feeding remains suppressed. Such suppression, however, is not limited to those behavioral options that are excluded to begin with. Rather, in a fight-or-flight situation both fight and flight, despite both being 'primed', both need to remain disabled until the decision is taken. Obviously, one solution here is lateral inhibition between the two behavioral options (including the use of inhibition to organize the switch between alternative functional configurations of a given network: Kristan, 2008). Thus, decision making will often require heavy inhibition, both to keep behavioral options 'off of the table', as well as inhibition between those options that are being pitted against or between network elements that are part of a combinatorial motor code. This implies that much of neuronal activity, be it excitatory or inhibitory, is related to being able to potentially do things without actually doing them – and *not* to processing along sensory-motor loops.

To conclude, there seems to be something so fundamental to the uncertainty in the world around us and in our sensory and cognitive faculties that just does not allow for a switchboard-type of brain and

that forces us to make decisions and take our chances. Still, the causal texture of the world seems sufficiently obvious and stable for reasoned action, and our bodily homeostasis sufficiently stable for personal consistency. It is this sphere of medium uncertainty where decision making and our personal lives are staged.

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I.3 Maggot learning and Synapsin function

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Abstract

Drosophila larvae are focused on feeding and have few neurons. Within these bounds, however, there still are behavioural degrees of freedom. This review is devoted to what these elements of flexibility are, and how they come about. Regarding odour-food associative learning, the emerging working hypothesis is that when a mushroom body neuron is activated as a part of an odour-specific set of mushroom body neurons, and coincidently receives a reinforcement signal carried by aminergic neurons, the AC-cAMP-PKA cascade is triggered. One substrate of this cascade is Synapsin, and therefore this review features a general and comparative discussion of Synapsin function. Phosporylation of Synapsin ensures an alteration of synaptic strength between this mushroom body neuron and its target neuron(s). If the trained odour is encountered again, the pattern of mushroom body neurons coding this odour is activated, such that their modified output now allows conditioned behaviour. However, such an activated memory trace does not automatically cause conditioned behaviour. Rather, in a process that remains off line from behaviour, the larvae compare the value of the testing situation (based on gustatory input) to the value of the odour-activated memory trace (based on mushroom body output). The circuit toward appetitive conditioned behaviour is closed only if the memory trace suggests that tracking down the learned odour will lead to a place better than the current one. It is this expectation of a positive outcome that is the immediate cause of appetitive conditioned behaviour. Such conditioned *search for reward* corresponds to a view of aversive conditioned behaviour as conditioned *escape from punishment*, which is enabled only if there is something to escape from – much in the same way as we only search for things that are *not* there, and run for the

emergency exit only when there *is* an emergency. One may now ask whether beyond 'value' additional information about reinforcement is contained in the memory trace, such as information about the kind and intensity of the reinforcer used. The *Drosophila* larva may allow developing satisfyingly detailed accounts of such mnemonic richness. If it exists.

1: Behavioural paradigms for associative learning in larval *Drosophila*

The brain is not the maggot's most impressive organ (Fig. 1). It contains an estimated order of magnitude fewer neurons than adult *Drosophila*, and is correspondingly smaller: The entire antennal lobe of a stage 3 larva fits into a single glomerulus of an adult fly (and a fly brain fits into the antennal lobe of a bee, we hesitate to add). Indeed, a larva's brain is considerably smaller than its salivary glands, betraying that one way or another much of brain function is devoted to food uptake, while a number of other occupations, such as flying, courtship, copulation, and egg-laying, obviously are of no concern to a larva. Thus, with the evolutionary 'outsourcing' of the feeding stage into a separate, larval form of life in the holometabolous insects, not only did various external structures such as wings, legs, and sex organs become dispensable, but a number of brain structures did as well, such as the central complex. Given the relatively slow locomotion of larvae, sensory systems can operate at massively reduced cell number. This is particularly striking in the visual system, which in the adult is comprised of approx. 13.000 neurons (2 x 6000 sensory neurons in the eyes, 3 x 80 photoreceptors in the ocelli and 2 x 6 in the Hofbauer-Buchner eyelet) as compared to 24 in larvae (2 x 12, Steller et al. 1987); we note that in line with our above comment the gustatory system is relatively well equipped in the larva with 80 (Python and Stocker 2002) to 90 (Colomb et al. 2007) neurons per body side. The maggot brain therefore *is* impressive - in numerical simplicity and functional 'focus'. Notably, within these bounds there still are behavioural degrees of freedom for the larvae. This review is devoted to what these elements of flexibility are, and how they come about. It aims at two goals: providing an overview of maggot learning as well as a detailed and comparative account of Synapsin function. This is because Synapsin provides an evolutionarily conserved experimental handle for unbroken chains of explanation between the behavioural and the molecular level of mnemonic processing.

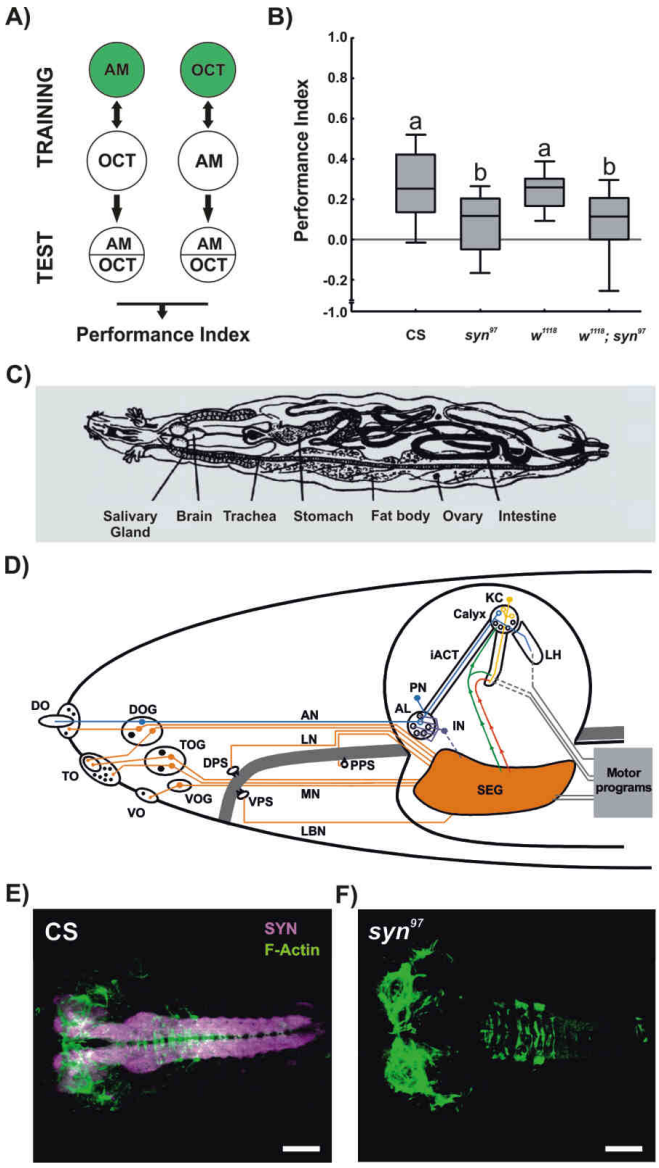

Figure 1

(A) The odour-sugar associative learning paradigm. Circles represent Petri dishes containing a sugar reward (fructose, green) or plain agarose (white). Groups of 30 larvae each are trained such that either *n*-amylacetate is rewarded and 1-octanol is not (AM+/OCT), or are trained reciprocally (OCT+/AM) (CAS numbers for AM and OCT: 628-63-7, 111-87-5). After three such training cycles, larvae are tested for their choice between AM *versus* OCT (for half of the cases, the sequence of training trials is reversed: OCT/AM+ and AM/OCT+). From the difference in preference between the reciprocally trained groups the performance index is calculated to quantify associative learning. For details, see Gerber *et al.* (2010).

(B) Associative function is reduced by about 50 % in syn^{97} mutant larvae relative to CS wild type larvae; this effect is also seen in the w^{1118} background. Note that associative function is equal in CS wild type and w^{1118} mutant larvae; this is important as one typically uses w^{1118} as a marker to keep track of transgenes when employing the Gal4-UAS technique. Box plots present the median as the bold line; box boundaries and whiskers present 25, 75 and 10, 90 % quantiles, respectively. Different lettering indicates significant differences in Mann-Whithney U-tests corrected for multiple comparisons. For behavioural controls see text.

(C) Body plan of larval *Drosophila*.

Figure 1 (continued)
(D) Overview of the cephalic chemosensory pathways of the larva. Olfactory sensory neurons (blue) project toward the brain hemispheres into the antennal lobe and then, via projection neurons (blue) toward both the mushroom body (yellow) calyx region and the lateral horn. Gustatory sensory neurons (orange) bypass the brain hemispheres and are collected in various regions of the subesophageal ganglion. The green and red arrows indicate pathways to short-circuit taste-driven aminergic reinforcement signals from the subesophageal ganglion toward the brain.
Abbreviations: AL: antennal lobe; AN: antennal nerve; DO/ DOG: dorsal organ/ dorsal organ ganglion; DPS: dorsal pharyngeal sensillae; iACT: inner antenno-cerebral tract; IN: antennal lobe interneurons; KC: Kenyon cells comprising the mushroom body; LBN: labial nerve; LH: lateral horn; LN: labral nerve; MN: maxillary nerve; PN: projection neurons; PPS: posterior pharyngeal sensillae; SEG: suboesophageal ganglion; TO/ TOG: terminal organ/ terminal organ ganglion; VO/ VOG: ventral organ/ganglion; VPS: ventral pharyngeal sensillae.
(D, E) Immunoreactivity against Synapsin (magenta) and F-Actin (green) in 3rd instar larval brains of a wild type (CS) and a syn^{97} mutant larva. Synapsin immunoreactivity is found throughout the neuropil area of brain and ventral nerve cord in the wild type, but is absent in syn^{97} mutant. Scale bars: 50 µm.
(B, E, F) based on data from Michels *et al.* (2011); (C) modified from Demerenc and Kaufmann (1972); (D) based on Stocker (2008).

We restrict ourselves to Pavlovian conditioning. In this form of associative learning, animals learn the predictive relation between two events, allowing them to behave according to the causal texture of the world (Dickinson, 2001). Regarding adult *Drosophila*, this research was pioneered by Benzer and colleagues (Quinn et al. 1974). In the presently used form (Tully and Quinn 1985), the paradigm presents flies with an odour (A) together with electric shock (-), while another odour is presented without shock (B). After such A-/B training, the animals are offered a choice between A and B (A vs B). Because A signals upcoming shock, the flies escape from A and/or toward the previously unpunished, safe odour B. In order to average out a possible innate preference for B, it is essential to run the experiment in a reciprocal manner (A/B-, test A vs B) in a second set of animals. Given that the two experimental groups differ in only the target parameter, namely the association between odours and shock, this allows one to calculate an associative performance index by comparing the A vs B choice after A-/B training to the A vs B choice after the reciprocal A/B- training (Tully 1984) (paradigms and findings not using such reciprocal design and thus potentially confounded by non-associative effects are not considered here). This paradigm was about a decade later supplemented with an appetitive version (A+/B, test A vs B as well as the reciprocal A/B+, test A vs B), where a sugar reward instead of shock punishment is used to induce a conditioned search for the sugar at the previously rewarded odour (Tempel et al. 1983). These two forms of learning attracted much experimental attention, and are, owing to the joint efforts of the *Drosophila* community, among the better understood study cases in the field of learning and memory to date (reviewed in Heisenberg

2003, Gerber et al. 2004, Margulies et al. 2005, McGuire et al. 2005, Schwärzel and Müller 2006, Keene and Waddell 2007, Heisenberg and Gerber 2008, Zars 2010, Davis 2011).

Five years after the seminal paper on adult *Drosophila*, a corresponding paradigm for odour-shock associative learning in larvae was introduced (Aceves-Pina and Quinn 1979), but compared with adult *Drosophila* the progress in understanding this form of learning has been slow (Tully et al. 1994, Khurana et al. 2009, Pauls et al. 2010a). Notably, it was as much as 25 years later that Scherer et al. (2003) introduced an appetitive version of this assay, using fructose as food reward (for a detailed manual see Gerber et al. 2010). Maybe thanks to the ecological validity of odour-food associations, this low-tech assay turned out to be robust and was adopted both in a number of research programs and in teaching at graduate, undergraduate as well as high school levels. Subsequently, low-concentrations of salt (approx. 0.5 M; Niewalda et al. 2008, Russel et al. 2011) were likewise found to be effective as reward, whereas high-concentrations of salt as well as quinine (Gerber and Hendel 2006, Niewalda et al. 2008, Selcho et al. 2009, Russel et al. 2011, Schleyer et al. 2011) and mechanosensory disturbances (Eschbach et al. 2011) can work as punishment. Last, but not least, an associative light-tastant learning paradigm is available (Gerber et al. 2004), but it yields a less favourable signal-to-noise ratio as compared with odour-tastant learning (possibly because it is less ecologically valid), and therefore has received less experimental attention (Kaun et al. 2007, von Essen et al. 2011). In the following sections, we sketch the circuitry and molecular mechanisms for the acquisition and storage of odour-reward memory traces, and will discuss how these memory traces, once established, are used for behavioural organization.

2: Chemobehavioural circuitry of larval *Drosophila*

In general, the behavioural repertoire toward odours is largely restricted to orienting movements and subsequent search or escape behaviour, dependent on the presumed nature of the odour source. In other words, animals use odours ‘to guess what’s over there’, to decide whether to search for or escape from the odour source, and to prepare for action in case the odour source is successfully tracked, or should escape fail. In order for this ‘guesswork’ to be as informed as possible, the olfactory sensory-

motor loop features stages of processing with an enormous potential to discriminate odours, as well as to attach acquired meaning to them.

In contrast, contact chemosensation serves to behave in immediate physical contact. Indeed, contact chemosensation is closely entangled with mechanosensation: Typically contact chemosensory sensilla in addition to chemosensory receptor neurons also harbour one mechanosensory neuron (Falk et al. 1976; Ishimoto and Tanimura 2004; see also Awasaki and Kimura 1997 for a developmental argument), serving to integrate the chemosensory 'what' with the mechanosensory 'where'. This may allow for fairly direct and often local sensory-motor loops in diverse contexts such as predation and defense, kin and/or nestmate recognition, aggression, the pursuit of courtship and copulation, oviposition and pupariation. Furthermore, cephalic and oral contact chemosensation organizes eating and drinking behaviour, and it is this 'taste' system that is of relevance in the present review.

The cellular architecture of the chemobehavioural system in *Drosophila* was revealed by the systematic analyses of the Stocker group over the past 30 years (reviewed in Stocker 1994, Stocker 2001, Vosshall and Stocker 2007, Stocker 2008) providing a basis for the ongoing work at the molecular and behavioural levels. It turned out that this architecture in the larva is similar to the one in adults, but with reduced cell numbers. In simplified terms, odours activate specific combinations of the 21 olfactory sensory neurons housed in the dome of the dorsal organ of each body side, largely dependent on the ligand profile of the receptor gene expressed in the respective cell (for detailed reviews and reference to the relevant original literature, as well as for exceptions to the 'rules of thumb' presented here, please consult Stocker 1994, Cobb 1999, Tissot and Stocker 2000, Gerber and Stocker 2007, Melcher et al. 2007, Vosshall and Stocker 2007, Stocker 2008, Cobb et al. 2009, Gerber et al. 2009). Each of these cells expresses one member of the *Or* receptor gene family (plus the *Orco* [CG10609] gene product [Vosshall and Hansson 2011] that is necessary for proper receptor trafficking and function), and projects to one glomerulus in the larval antennal lobe; these glomeruli can be identified by position. In turn, each receptor gene is expressed by one olfactory sensory neuron, and each glomerulus receives input from one of these sensory neurons. Whether the receptors of the *Ir* gene family (Benton et al. 2009) play a role for larval chemosensation remains to be investigated.

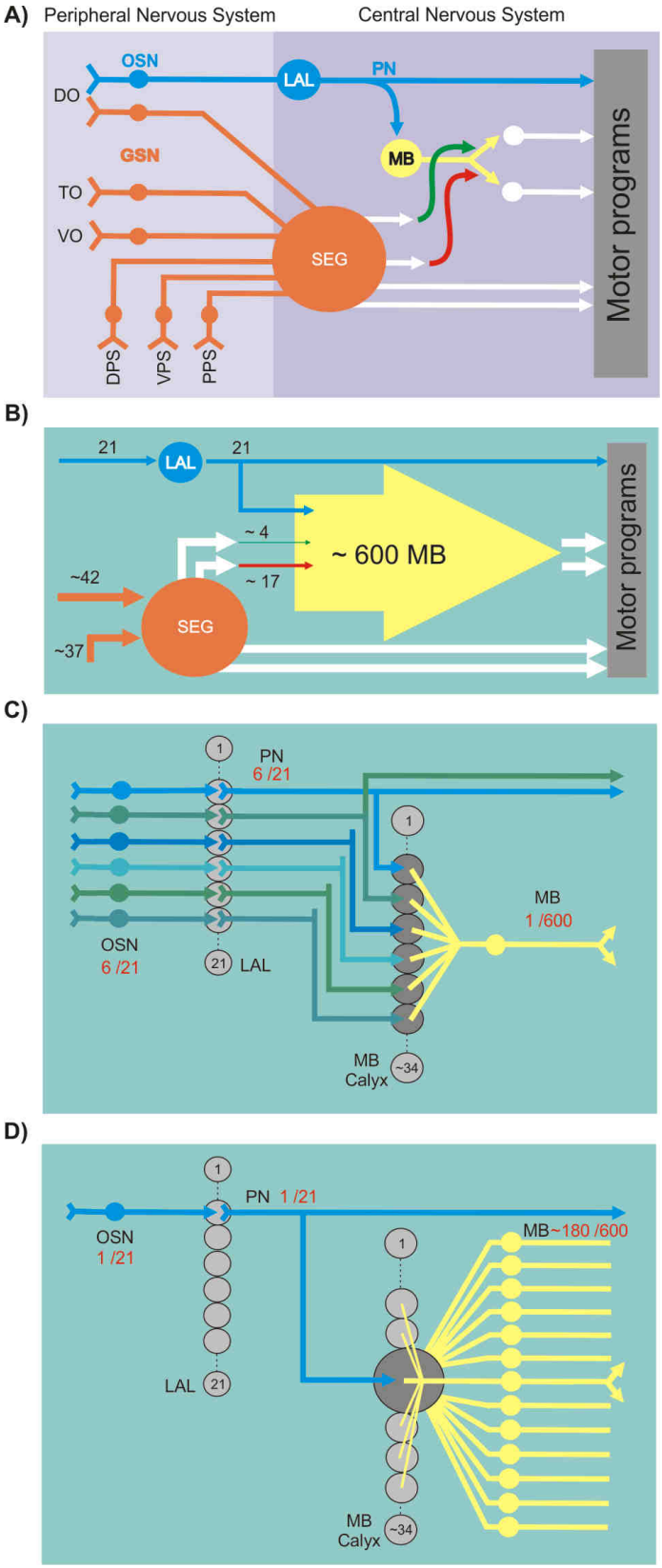

Figure 2
Schematic representation of the cephalic olfactory (blue) and gustatory (orange) pathways of the larva, and their downstream elements, in particular the mushroom body neurons (MB, yellow). White fill indicates unknown or relatively ill-characterized parts of the circuit.
(A) Olfactory sensory neurons (OSN) housed in the dome of the dorsal organ (DO) terminate in the larval antennal lobes (LAL). From the LAL, projection neurons (PN) deliver olfactory information toward (pre)motor circuits via two routes, directly via the lateral horn (not shown) sufficient for innate olfactory behaviour, and through a detour via the MB. Gustatory sensory neurons (GSN) from the bulge of the DO, the terminal and ventral organs (TO, VO), as well as from the internal dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, PPS) terminate in the subesophageal ganglion (SEG); note that DO, TO, VPS, and likely the DPS as well, contain non-gustatory neurons. From the SEG gustatory processing follows two routes. One route connects toward innate gustatory behaviour. The second route consists of modulatory octopaminergic/tyraminergic (OA, green) and dopaminergic (DA, red) neurons. These have a number of targets (Selcho et al. 2009, 2012); here we show the ones that likely relay reinforcement information toward the mushroom body neurons; note that OA and DA innervate different subcompartments of the mushroom bodies. The net effect of the OA (as covered by TDC-Gal4) system is rewarding, and the net effect of the DA system (as covered by TH-Gal4) is punishing (see last paragraph of section 2 for important caveats concerning this dichotomy). Thus, the mushroom body neurons are a likely cellular site of convergence of olfactory processing and reinforcement signalling. Downstream of the MB, conditioned olfactory behaviour is organized (for details of the organization of conditioned behaviour, see Fig. 6). Note that the LAL also harbours intrinsic neurons and neurons to connect LAL and SEG (not shown).
(B) Simplified version of (A), with the size of the elements drawn according to the number of known involved cells; numbers are per hemisphere.
(C, D) Convergence-divergence relationships of the PN-MB interface at the calyx of the mushroom bodies. Up to six of the 21 OSN-PN input lines converge onto a given MB neuron (C). In turn, within its cognate MB glomerulus (enlarged for clarity) a given PN diverges to up to 180 of the 600 MBs (D) (for details of this number game see Gerber and Stocker [2007]). Note that the presented numbers are based on the simplified assumption that MB neurons receive input from six PNs (only for one MB neuron these six connections are drawn) although MBs actually are heterogeneous, connecting to 1-6 PNs; also note that it is unknown how many PNs need to be active to drive an MB.

In the antennal lobe, lateral connections between the glomeruli (Thum et al. 2011; this study also contains the description of hitherto unrecognized neurons connecting antennal lobe and subesophageal ganglion) shape the pattern of activation that then is carried forward by the output elements of the antennal lobe, the projection neurons. The projection neurons typically receive input in one glomerulus; correspondingly, a given antennal lobe glomerulus harbours the dendrites from typically one projection neuron. Thus up to this level in the circuit, the olfactory system lacks cellular redundancy. Notably, the axons of the projection neurons are branched: One axon collateral conveys odour information directly toward the lateral horn and presumably onto pre-motor circuits. The other collateral establishes a detour of olfactory information flow via the mushroom bodies. In the input region of the mushroom bodies, the calyx, the coding space is massively expanded. And then is massively reduced. That is, each of the approx. 21 projection neurons typically innervates one of the approx. 35 mushroom body glomeruli (thus, some mushroom body glomeruli apparently receive input from neurons other than the olfactory projection neurons). Different from the situation in adults, many of the mushroom body glomeruli can be identified by position, revealing a reliable relation between the antennal lobe glomerulus in which a projection neuron receives input and the mushroom body glomerulus to which it provides output. This allows the majority of the projection neurons to be individually identified. Each of the strikingly many mature mushroom body neurons (approx. 600, of which 250-300 are embryonic-born and may be of particular relevance for associative function: Pauls et al. 2010b) receives convergent input in an apparently random set of 1-6 mushroom body glomeruli and hence from 1-6 projection neurons (Fig. 2C). Dependent on how many of these inputs need to be activated to drive the mushroom body neuron, it thus may sample up to a third of the larval odour space. In turn, each projection neuron diverges to 30-180 mushroom body neurons (for details of this approximation see Gerber and Stocker 2007), thus reporting to a third of the mushroom body coding space (Fig 2D). Assuming that mushroom body neurons need coincident excitatory input from more than one projection neuron to fire, this combined convergent-divergent architecture allows for combinatorial coding and can enhance small differences in input (for a review of temporal aspects of olfactory coding see Laurent et al. 2001). Output from the mushroom bodies is drawn by relatively few neurons (a reasonable guess based on Pauls et al. 2010b is that these may be an order of

magnitude fewer neurons than mushroom body neurons), which each sample many if not all mushroom body neurons, and which project onto pre-motor circuitry. The output side of the mushroom bodies thus may be viewed as a sensory-motor 'watershed': An enormous combinatorial space to code for odour identity is 'reformatted' onto rather few pre-motor channels.

It is important to note that the premotor systems, ill-characterized as they are, thus receive two kinds of olfactory signal, one from the direct pathway, and one via the mushroom body detour. As will be argued below, the direct pathway mediates innate behaviour such as olfactory choice in experimentally naïve animals, while the mushroom body detour mediates learned behavioural tendencies toward odours.

Compared to the olfactory system, the taste system is less well understood (see reviews by Gerber and Stocker 2007, Melcher et al. 2007, Cobb et al. 2009, Gerber et al. 2009, and references therein). It comprises approx. 2 x 80-90 gustatory sensory neurons, located in six paired sensory structures, three of them external (terminal organ, ventral organ, and the bulge of the dorsal organ) and three internal (ventral, dorsal, and posterior pharyngeal sense organs). The gustatory sensory neurons bypass the brain *proper* and project to the subesophageal ganglion in a way that depends on the receptor gene(s) expressed and their sense organ of origin (Colomb et al. 2007). Taste information then is relayed to modulatory neurons which detour toward the brain and/or to the ventral nerve cord, as well as to (pre-)motor circuitry presumably in the ventral nerve cord to mediate taste-related behaviours. Such behaviours include preference for sugars and low salt concentrations, as well as avoidance of high salt concentrations and 'bitter' substances; both sugars and salt stimulate feeding at relatively low, but suppress feeding at higher concentrations. However, despite significant recent progress (e.g. Kwon et al. 2011), the principles of circuit organization underlying taste-mediated behaviours in the larva remains unresolved. In particular, we lack a comprehensive view of the entanglement of the taste system with mechanosensation and thus of the 'what?' and 'where?' of contact chemosensory processing, and of the role of chemosensory input from the gut and/or of metabolic feedback onto brain and behaviour. Also, one can at present only suspect that, in analogy to the situation in adult flies, the ability of the larvae to discriminate between e.g. different kinds of sweetness or different kinds of bitterness is limited, and that some of these limits are established

already at the level of the sensory neurons (for discussion see Gerber et al. 2009). In other words, in the gustatory system the sensory-motor 'watershed' appears to be pushed relatively far out toward the sensory periphery, categorizing inputs early on into behavioural categories. This organization contrasts with the olfactory system, which features stages with an enormous capacity to discriminate odours, and the flexibility to tell apart or categorize between odours, depending on the task (Mishra et al. 2010).

In any event, from the discussion above it appears as if there is no connection between olfactory and taste pathways (but see Thum et al. 2011). How, then, can odours be associated with tastants? In a seminal paper, Hammer (1993) identified the octopaminergic VUMmx1 neuron in the honeybee, which receives gustatory input likely in the subesophageal ganglion and provides output to the antennal lobe, the mushroom body and the lateral horn. This neuron thus 'short-circuits' taste with olfactory pathways, and is sufficient to mediate the rewarding function of sugar in honeybee olfactory learning. This neuron exists in adult (Busch et al. 2009) and larval *Drosophila* as well (Selcho et al. 2012). In larvae the net effect of driving subsets of octopaminergic/tyraminergic neurons (TDC-Gal4) can substitute for reward in olfactory learning, while driving subsets of dopaminergic neurons (TH-Gal4) can substitute for punishment (Schroll et al. 2006). Current research suggests that the mnemonic function of the neurons contained in these sets is not restricted to such reinforcement processing during training, though. For example, in adult *Drosophila*, the dopaminergic MB-MP neurons seem to carry visceral and/or metabolic feedback information ('satiety state') toward the mushroom body to effectively inhibit mushroom body output (Krashes et al. 2009). Also, it turned out that some dopaminergic and conceivably also octopaminergic/tyraminergic neurons transgress a simple appetitive-aversive dichotomy (Schroll et al. 2006 [loc. cit. Supplemental Material], Honjo and Furukubo-Tokunaga 2009, Selcho et al. 2009, Liu et al. 2012) (for corresponding data in mice see Kravitz et al. 2012). What is important for the present discussion is that the convergence of the olfactory pathway is not with the gustatory pathway itself, but with a modulatory signal branching off of the gustatory sensory-motor loop. At which point(s) in the circuit does such memory trace-inducing convergence take place- or in other words where in the circuit are odour-reward memory traces formed?

3: Synapsin function: Background

We used a three-step strategy toward memory trace localization (Gerber et al. 2004, Heisenberg and Gerber 2008): (i) find a gene which, when mutated, impairs neuronal plasticity and odour-reward learning; (ii) test in which parts of the chemobehavioural circuits restoring this gene is sufficient to restore associative function; (iii) test whether these sites of sufficiency also are sites where the expression of that gene is necessary. As a candidate gene for such an analysis, we chose to focus on *synapsin* (Fig. 3 displays the *synapsin* gene of *Drosophila*).

Synapsins are highly abundant soluble neuronal phosphoproteins, located in nerve terminals and associated with the cytoplasmic surface of synaptic vesicle membranes (for reviews of the original literature please see: Benfenati [ed.] 2011, Cesca et al. 2010, Fornasiero et al. 2010, Fdez and Hilfiker 2006, Ferreira and Rapoport 2002, Hilfiker et al. 1999, Kao et al. 1999, Greengard et al. 1993, Südhof 1995, DeCamilli et al. 1990, Südhof et al. 1989). Vertebrates posses a three-member family of *synapsin* genes, while in invertebrates such as *Drosophila* only one *synapsin* gene is found (Klagges et al. 1996); from each gene, typically multiple protein isoforms are produced by alternative splicing. Synapsins are part of the presynaptic molecular network to fine-tune synaptic output, in particular to regulate the balance between the different presynaptic vesicle pools in a phosphorylation-dependent manner. That is, there likely are at least three functionally distinct (yet not necessarily spatially segregated) presynaptic vesicle pools (Rizzoli and Betz 2004) (for a novel perspective regarding these pools, see Denker et al. 2011ab): (i) a reserve pool bound to actin filaments and not immediately available for release; (ii) a readily-releasable pool of docked vesicles at the presynaptic membrane primed for immediate exocytosis; (iii) the cycling pool, comprised of those vesicles engaged in the exo-endocytic cycle. Synapsins can bind to both synaptic vesicles and to actin, the latter in a phosphorylation-dependent way. Seminal work of Greengard and colleagues suggested a role of Synapsin in non-associative plasticity. Their analyses indicate that phosphorylation of Synapsin reduces its affinity to actin and thus recruits vesicles from the reserve pool for subsequent rounds of exocytosis. From a comparative perspective, it is noteworthy that domains A and C at the N-terminal side of the protein are shared by all known Synapsin isoforms, whereas additional domains (D to J) at the C-terminal side are less conserved. Phosphorylation sites in the A domain include an

evolutionarily highly conserved PKA/CaM I/IV kinase consensus site; also, within this A-domain a candidate membrane-binding region was identified. The C domain likewise contains such a candidate membrane-binding region, while other sites within the C-domain appear responsible for binding to actin filaments. These insights into Synapsin function were subsequently enriched in a number of ways (for detailed overviews, see the contributions in Benfenati [ed.] 2011):

(i) Concommittant with the discovery of the requirement of Synapsin in non-associative short-term plasticity in *Aplysia* (Humeau et al. 2001), it was shown that serotonin induces Synapsin phosphorylation in *Aplysia* (Angers et al. 2002), likely via the cAMP-PKA pathway (Fiumara et al. 2004, see also Dolphin and Greengard 1981) and/or the MAP kinase pathway (Giachello et al. 2010), and that these processes impact non-associative short-term plasticity (Fioravante et al. 2007, Doussau et al. 2010, Giachello et al. 2010; for a recent related study in the mouse see Valente et al. 2012). Strikingly, Hart et al. (2011) lately reported that the *synapsin* gene of *Aplysia* features a CRE-recognition site, that serotonin induces *de novo* synthesis of Synapsin via the CREB1 pathway, and that this is required for non-associative long-term plasticity.

(ii) The description of the crystal structure of a recombinant Synapsin C-domain revealed a similarity to ATP-binding enzymes (Esser et al. 1998) and it was then shown that recombinant C-domains of Synapsin I, II and III bind ATP *in vitro* (Hosaka et al. 1998[a,b]). Interestingly, ATP-binding by Synapsin I but not by Synapsin II is Ca^{2+}-dependent, while Synapsin III is inhibited by Ca^{2+}.

(iii) Additionally, the crystal structure of the C-domain suggested a possible dimerization of Synapsins (Esser et al. 1998), which was confirmed by Hosaka et al. (1999). This process is promoted by ATP and Ca^{2+} (Brautigam et al. 2004). Given that actin depolymerization apparently does not disrupt vesicle clusters (Sankaranarayanan et al. 2003), whereas Synapsin 'neutralization' by antibodies does (Pieribone et al. 1995), it is speculated that Synapsins may bind vesicles not only to the actin cytoskeleton but to each other as well (Bykhovskaia 2011, Shupliakov et al. 2011).

(iv) Krabben et al. (2011) identified an ALPS motif in the B-domain which, when mutated, affects the association of Synapsin with curved membranes, including synaptic vesicles, suggesting a novel way of how Synapsin acts in regulating vesicle function.

(v) Additional implications of Synapsins, partially mediated by MAP kinases, include developmentally relevant aspects such as neurite elongation, synaptogenesis and synapse maturation (Valtorta et al. 2011, Fornasiero et al. 2010).

Last but not least, the mosaic expression pattern of different Synapsin isoforms in the vertebrate brain early on suggested a functional heterogeneity not only between domains and phosphorylation sites of Synapsin, but also between isoforms. Indeed, *synapsin II*, but not *synapsin I* mutant mice were later reported to be impaired in associative learning (Silva et al. 1996). Mice lacking all three *synapsin* genes perform poorly in a number of tested reflexes and their ability to hang from a suspended wire; they also show seizures upon disturbance, reduced piloerection, and difficulties maintaining balance (Gitler et al. 2004). Regarding mnemonic function, these animals also performed poorly in a test for spatial memory, without reported task-relevant disturbances in motivation or motor ability. In humans, mutations in *synapsin* genes can apparently entail severe neurological, behavioural, and/or psychiatric phenotypes (Garcia et al. 2004, Fassio et al. 2011, Porton et al. 2011).

Thus, the *synapsin* genes contribute to bewilderingly many functions, dependent on which particular species and gene is looked at, which isoform, domain or site is considered, how the respective protein is embedded into the local molecular network at a given presynapse, and the way the respective neuron is integrated into a functional circuit and ultimately into behavioural task(s). In other words, there does not seem to be anything like *the* role of Synapsin. Assigning one particular behavioural role for a particular gene, isoform and molecular interaction, at a particular synapse of a given circuit obviously does not rule out other roles in other contexts. In turn, the discussion below will show that for a precisely defined behavioural task, a cellular site and a molecular mode of Synapsin action can be assigned with satisfying precision.

4: Synapsin function: *Drosophila*

The presence of Synapsin-like immunoreactivity in invertebrates was known since the mid-80s, but the first invertebrate gene coding for Synapsin (*synapsin, syn*, CG3985) was identified from *Drosophila* only in 1996 in a seminal project of the Buchner group to identify brain specific proteins (Fig. 3) (Klagges et al. 1996, Hofbauer et al. 2009). The intron/exon structure of this gene exhibits similarity to *synapsin* genes of vertebrates (Klagges et al. 1996, Godenschwege et al. 2004) and as in many of these genes, the gene for the tissue inhibitor of metalloproteinase (*timp*, CG6281) is nested within the *syn* gene on the antiparallel DNA strand (Pohar et al. 1999). Alternative splicing of the primary transcript produces two *synapsin* transcripts, leading to two protein isoforms with relatively low weight (70-80 kDa); additionally, a full-length protein (~143 kDa) can be generated via read-through of an *amber* stop codon into the second open reading frame (Klagges et al. 1996, Nuwal et al. 2011); indeed, polyclonal mouse sera against this ORF2 specifically detect the 143 kD band (Klagges et al. 1996). The mouse monoclonal antibody SYNORF1 binds in the conserved C-domain of Synapsin (epitope LFGGMEVCGL, Fig. 3) (Godenschwege et al. 2004); it has turned out as a particularly useful tool, because of its high affinity binding, its suitability in histology and Western blotting, and the fact that it can be used across species. Notably, the mentioned antisera against the full-length protein reveal staining patterns not obviously different from that of SYNORF1.

The *Drosophila* Synapsin protein contains domains homologous to the N-terminal A-domain and the central, actin- and vesicle-binding C-domain (50 % identity, 89% similarity of amino acids); additionally, the prolin-rich C-terminus shows similarity to the E domain found in a-type vertebrate synapsins. *Drosophila* Synapsin contains a number of predicted phosphorylation sites, including the evolutionarily conserved PKA/CamK I/IV consensus motif RRFS at Ser^{6} (henceforth called PKA-1 site), the evolutionarily non-conserved PKA/CamK I/IV consensus motif RRDS at Ser^{533} (henceforth called PKA-2 site), and additionally suspect sites for other kinases, i. e. CamK II, prolin-dependent kinase, and PKC (Nuwal et al. 2011). *Drosophila* Synapsin is expressed exclusively in most if not all neurons, starting at mid-embryogenesis. On a subcellular level, the SYNORF1 antibody reveals expression in type-Ib and type-Is boutons of larval motorneurons, but not in type-II and -III boutons (Klagges et al. 1996, Godenschwege et al. 2004). This situation, together with the role of Synapsins in

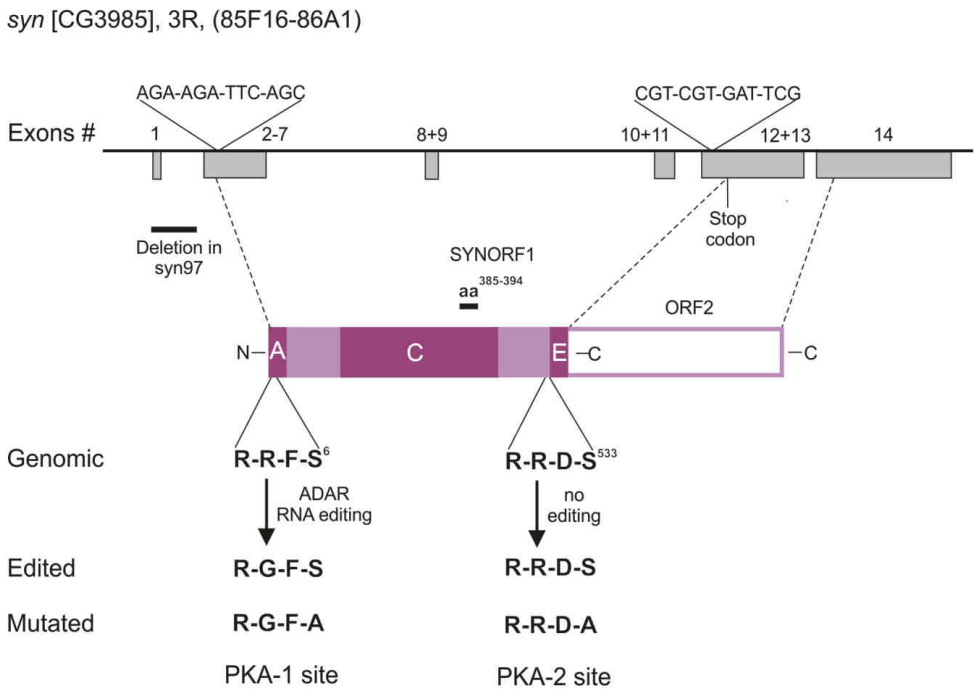

Figure 3
Genomic organization of the *synapsin* locus and organization of the Synapsin protein. Position and size of the *syn*^{97} deletion at the regulatory region up to the first intron is indicated. The exons are translated into several protein isoforms (two short isoforms of 70-80 kDA [magenta-filled boxes] and a 143 kDa full-length isoform [ORF2]). In these proteins, the N-terminal A-domain and the centrally located C-domain are highly conserved between vertebrates and flies. The C-terminus contains a prolin rich region and the less conserved E-domain; amino acids 385-394 feature the LFGGMEVCGL epitope of the SYNORF1 mouse monoclonal antibody. Superimposed are the locations of consensus sites (R-R-X-S) for PKA and CaMK I/IV and their amino acid sequences based on the genomic codons. RNA-editing at the PKA-1 site changes R-R-F-S to R-G-F-S on the protein level. The rescue construct used in the two leftmost panels of Fig. 4 A uses a UAS-construct to express the 'Edited' motif, whereas Fig. 4 A, rightmost panel, uses the 'Mutated' motifs.
Based on Diegelmann *et al.* (2006).

synaptic function and plasticity in mammals and molluscs (see above) prompted the question of the function of Synapsin in *Drosophila*. Toward this end, the *syn*^{97} mutation was used which shows a deletion including promoter regions, the first exon and part of the first intron of the *synapsin* gene (Fig. 3). Using SYNORF1 as well as a mouse polyclonal anti-synapsin antiserum it was shown that the deletion leads to a complete lack of the Synapsin protein (adults: Godenschwege 1999, Godenschwege et al. 2004; larvae: Michels et al. 2005, 2011). After removing the *white*^{1118} mutation (*white*, CG2759, which is used as genetic marker) by repeated outcrossing to wild type, *syn*^{97} mutant flies were found to be viable, fertile and normal in a number of simple behavioural tasks (initial olfactory jump response, basic optomotor response, basic walking parameters), as well as in presynaptic structure at the larval neuromuscular junction (i.e. the number of synaptic boutons, integrity of the T-shaped synaptic ribbon, number of vesicles immediately close to it, structure of the subsynaptic reticulum) and in basic

transmission at this synapse (Godenschwege et al. 2004). Interestingly, the notion of Synapsin maintaining vesicles in place is consistent with the finding that the synaptic vesicles in the boutons of motoneurons are distributed over larger areas in syn^{97} mutant larvae than in wild type; this effect can be phenocopied by motoneuron stimulation in wild type larvae (Akbergenova and Bykhovskaia 2010; see also Angers et al. [2002] for related findings on Synapsin dispersal upon serotonergic activation in *Aplysia*). Fittingly, Denker et al. (2011a) found that vesicles in syn^{97} mutants are more likely to be part of the non-reserve pools, in turn implying that Synapsin maintains the reserve pool.

Importantly in the current context, Godenschwege et al. (2004) found that in arguably more demanding and/or complex behaviour assays (ethanol tolerance, visual object fixation, conditioned courtship suppression, wing beat frequency during sustained flight, habituation of the olfactory jump response, performance in the heat-box conditioning paradigm [and in a paradigm where flies learn about the nutritional value of food: Fujita and Tanimura 2011]), the syn^{97} mutant does show phenotypes. Critically, the syn^{97} mutation entails an approx. 25 % decrease in odour-shock associative function, but leaves intact task-relevant odour processing, shock processing and the respective behavioural faculties to behave toward these stimuli (the mutants also behave normally to odours after training-like exposure to either odours alone or to shock alone: Knapek et al. 2010). This prompted us to test the syn^{97} mutant for associative function at the larval stage, too.

In larval syn^{97} mutant animals, odour-sugar associative function is reduced by about half (Fig.s 1B, 4; Michels et al. 2005, 2011), an effect that can be phenocopied by reducing Synapsin levels throughout development by transgenically expressing RNAi with elav-Gal4 as driver (Michels et al. 2011), while all behavioural control experiments (olfactory preference, sugar preference, susceptibility to training-like odour- or sugar-exposure) did not reveal a phenotype (Michels et al. 2005). As the syn^{97} mutation thus specifically affects associative function, we wondered when, in which cells and by which molecular mechanism Synapsin would function.

The reduced associative faculties of syn^{97} mutant larvae in the odour-sugar paradigm can be rescued by acutely restoring Synapsin throughout the nervous system (elav-Gal4; tub-gal80^{ts}; UAS-syncDNA, syn^{97}). Locally restoring Synapsin in the mushroom bodies is sufficient to fully restore associative ability (mb247-Gal4, UAS-syncDNA, syn^{97}), whereas restoring Synapsin in the projection

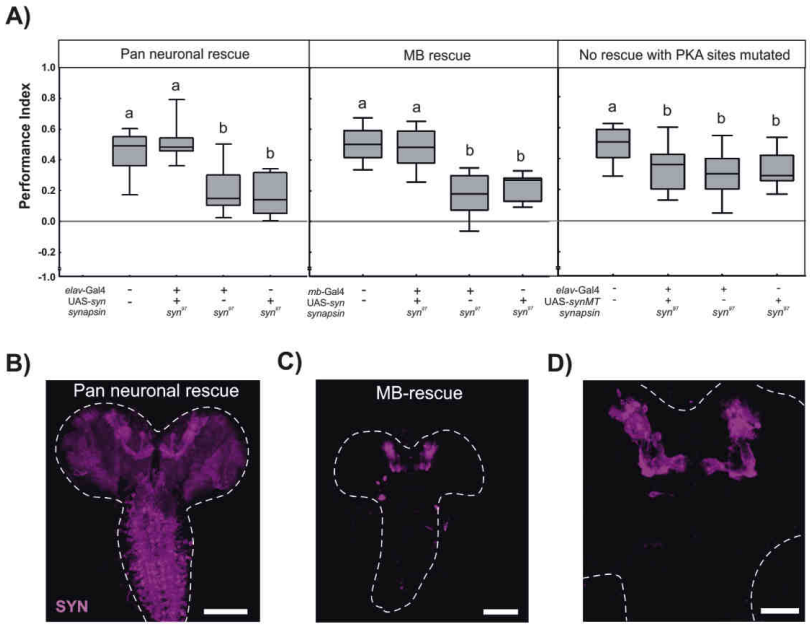

Figure 4
(A) The defect of the *syn*^{97} mutant in associative function is fully restored using a pan neuronal driver line in combination with a normal UAS-syn-cDNA construct (left panel, corresponding to the 'Edited' site in Fig. 4); the same is observed with acutely induced Synapsin expression (see text). A rescue is also achieved if Synapsin expression is restored only within the mushroom bodies (MB) (middle panel), but not when Synapsin is restored in the projection neurons, or outside the MBs (see text). No rescue is seen, either, by using the same MB-driver line but in combination with a UAS strain to express a version of Synapsin with both PKA consensus sites mutationally inactivated (right panel, corresponding to the 'Mutated' sites in Fig. 4). Other details as in Fig. 1.
(B- D) Immunreactivity against Synapsin (magenta) in the brains of the indicated genotypes. In (D), a close-up of (C) is shown. Scale bars: 50µm (B,C), 25µm (D).
Based on data from Michels *et al.* (2011).

neurons is not (either GH146-Gal4 or NP225-Gal4; UAS-syncDNA, *syn*^{97}) (in the two beforementioned cases and in all following cases, rescues were performed non-acutely, i.e. without tub-gal80^{ts}). We note that in cases where no rescue is observed, it is not only necessary to test by immunohistochemistry whether the transgene of interest is indeed properly expressed, but also whether the Gal4-driver element *per se* may have an effect in the experimental animals, such that an actually successful rescue is obscured; in the study of Michels et al. (2011), no such effects of the Gal4-driver elements were observed in any of the cases of lack-of-rescue. In any event, if Synapsin is restored in wide areas of the brain excluding the mushroom bodies, learning ability is not restored, either (elav-Gal4; mb247-Gal80; UAS-syncDNA, *syn*^{97}), suggesting Synapsin in the mushroom body may also be necessary for proper associative function. This latter conclusion of necessity remains tentative, however, because the mb247-Gal80 construct leads to Gal4 suppression also outside the mb247-Gal4 expression pattern and even outside of the mushroom bodies. Also, blocking synaptic

output of mb247-Gal4 positive neurons with *shibire*^{ts} (Kitamoto 2001) reportedly does not impair odour-sugar mnemonic processing (Pauls et al. 2010b), while broader mushroom body expression, arguably in particular in embryonic-born mushroom body neurons, does (201Y-Gal4, NP1131-Gal4). Thus, a Synapsin-dependent memory trace is located in the mushroom bodies, and this may turn out to be the only (i.e. necessary) site where such a trace is established in this particular paradigm. These findings prompted an enquiry into the molecular mode of action of Synapsin in memory trace formation, in particular into its possible role as a target of the AC-cAMP-PKA cascade. Indeed, with respect to odour-shock associative function in adult flies, Knapek et al. (2010) found that the *syn*^{97} mutation is non-additive with *rut*^{2080}, a hypomorphic mutation in the *rutabaga* gene (CG9533) coding for a type I adenylate cyclase.

The role of the AC-cAMP-PKA cascade for olfactory short-term memory has been intensely studied in adult flies (Tomchik et al. 2009, Gervasi et al. 2010, and references therein). The *rutabaga* type I adenylyl cyclase acts as molecular coincidence detector between the reinforcer and the olfactory activation of the mushroom body neurons: On the one hand, the odour leads to presynaptic calcium influx, and hence to an activation of calmodulin, in that subset of mushroom body neurons that is activated by this odour. On the other hand, the reinforcer activates aminergic neurons and hence the respective G-protein coupled amine receptors in many if not all mushroom body neurons. In the larva, an octopaminergic/tyraminergic set of neurons, defined by TDC-Gal4, carries a net rewarding effect, whereas a dopaminergic set of neurons, defined by TH-Gal4, carries a net punishing effect (Schroll et al. 2006) (see final paragraph of section 2 for qualifying detail). Those subsets of aminergic neurons which target the mushroom bodies likely each connect to most if not all mushroom body neurons, with octopaminergic/tyraminergic and dopaminergic neurons innervating different domains of the mushroom bodies (Pauls et al. 2010b, Selcho et al. 2012). Critically, it is only by the simultaneous activation of the calmodulin (‘odour’) and G-protein pathways (‘reward’ or ‘punishment’, respectively), that the AC is substantially activated. Thus it is only in those mushroom body neurons which receive both odour and reinforcement activation that cAMP levels and PKA activity are boosted, and the respective protein substrates get phosphorylated. However, the behaviourally relevant substrates of PKA in flies remained obscure. Our working hypothesis was that one of these PKA

substrates may be Synapsin, such that PKA-mediated phosphorylation of Synapsin recruits reserve-pool vesicles. Thus, a subsequent presentation of the learnt odour would result in enhanced output from an odour-specific set of mushroom body neurons, and ultimately in conditioned behaviour toward the learnt odour (Fig. 5). We therefore reasoned that transgenic mushroom-body expression of a Synapsin protein that carries dysfunctional PKA-sites (mb247-Gal4, UAS-syncDNA-PKA$^{S6A/S533A}$, *syn*^{97}) will not be able to rescue the *syn*^{97} associative defect. This is indeed the case (Michels et al. 2011).

Interestingly, the evolutionarily conserved PKA-1 site, but not the non-conserved PKA-2 site, undergoes ADAR-dependent pre-mRNA editing (Diegelmann et al. 2006). This manifests itself in a discrepancy between the genomic sequence and the sequence of at least the majority of mRNAs, and ultimately the amino acid sequence of the protein: The amino acid motif RRFS which should be optimal for phosphorylation by PKA is altered via RNA editing into RGFS, in both larval and adult flies. This edited RGFS form of Synapsin can hardly be phosphorylated, at least in an *in vitro* study using bovine PKA (Diegelmann et al. 2006). It should now be interesting to see *in vivo* whether and how RNA editing at Ser^{6} affects the efficacy of phosphorylation by PKA and/or other kinases, and how such fine regulation of phosphorylation impacts associative function. Along these lines we note that, in order to restore the wild type situation, the rescue experiment displayed in Figure 4A (left and middle panels) uses the edited form of Synapsin and that it does restore the defect in associative function (see above).

As a working hypothesis, we thus propose that a Synapsin-dependent memory trace underlying attraction to the conditioned odour in the larva is localized within the mushroom body neurons. Whenever a mushroom body neuron, as a part of an odour-specific subset of mushroom body neurons, is activated coincidently with an aminergic reinforcement signal, the cAMP-PKA cascade is triggered. One substrate of this cascade is Synapsin. Phosporylation of Synapsin likely by PKA but possibly also by other kinases ensures an alteration of synaptic strength between this mushroom body neuron and its target(s). If the trained odour is encountered again at test, the same odour-specific subset of mushroom body neurons is activated again, such that now their modified collective output toward their target

neuron(s) allows conditioned behaviour. In other words, the memory trace for the association of an odour with a reward consists in the pattern of changed synaptic weights across the array of the mushroom body neurons. But how, actually, is conditioned behaviour organized?

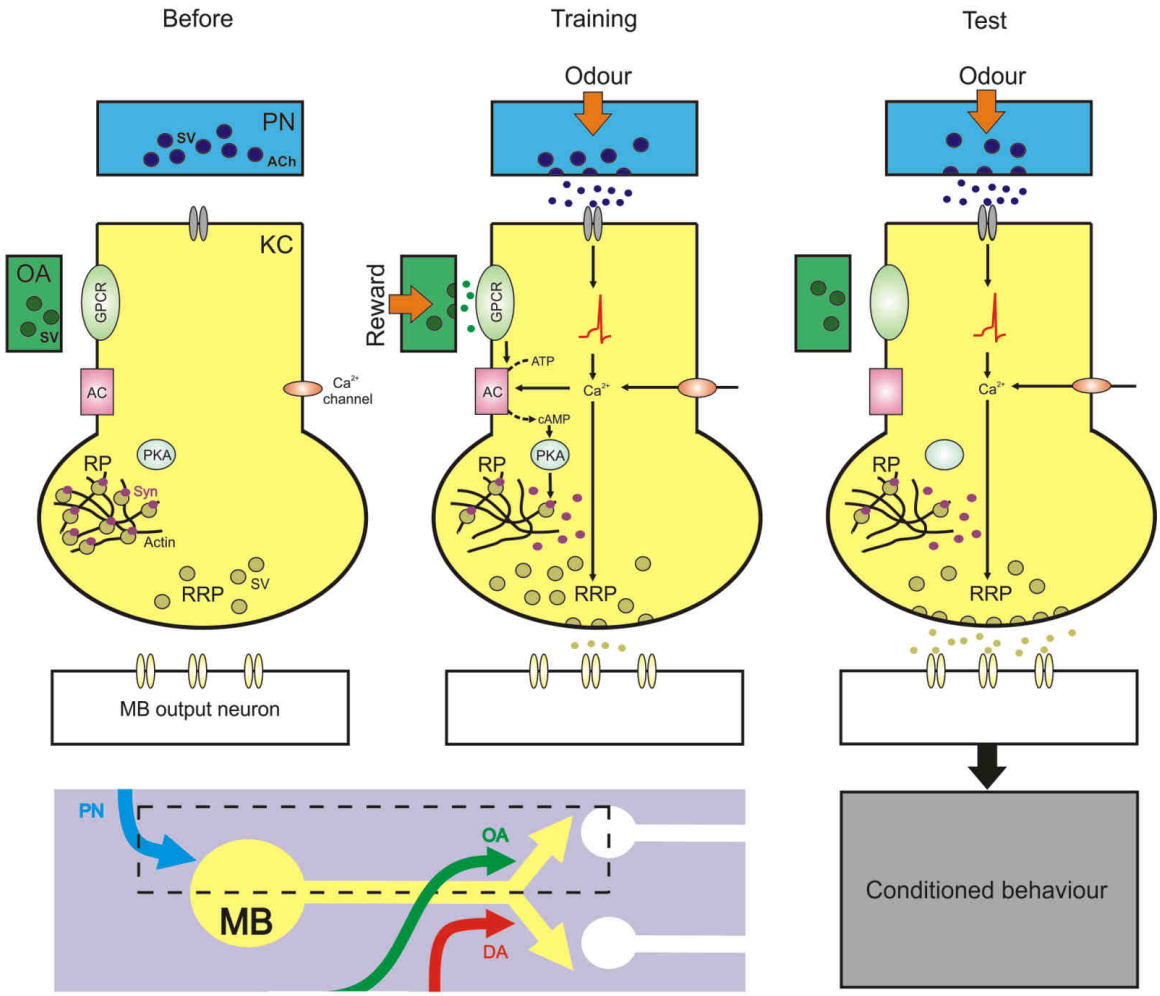

Figure 5
Working hypothesis of the role of Synapsin in memory trace formation as a target of the AC-cAMP-PKA pathway. Sketched are the molecular events at the synapse before, during and after paired presentations of odour and sugar reward. The odour evokes action potentials in an odour-specific subset of mushroom body neurons, and hence opening of voltage-gated presynaptic Ca^{2+} channels. On the one hand, this leads to fusion of synaptic vesicles (SV) from the readily relaseable rool (RRP). On the other hand, the adenylate cyclase (AC) is activated by coincident Ca^{2+} and G-protein signaling, the latter induced by sugar-evoked release of an aminergic reinforcement signal (for details see text) and hence activation of the respective G-protein coupled amine receptors (GPCR). cAMP has a number of intracellular effects including activation of protein kinase A (PKA). PKA in turn also has many targets, including Synapsin which contains two PKA/ CaMK I/IV consensus sequences, as well as consensus sequences for other kinases. Synapsin tethers vesicles to the actin cytoskeleton, thereby maintaining a reserve pool (RP) of vesicles. Upon activation by kinases (e.g. PKA) Synapsin gets phophorylated and lowers its affinity to the cytoskeleton, allowing for recruitment of synaptic vesicles for later release; note that RP and RRP are functionally yet not necessarily spatially segregated. If the learned odour is encountered again, the additionally recruited vesicles support potentiated output from the mushroom body neuron, and ultimately conditioned behavior. Note that this display refers to odour-reward associations only; odour-punishment associations likely are established in functionally separated domains of the mushroom body neurons and a distinct aminergic punishment signal (Fig. 2 A and inset at lower left of this figure; the stippled box in the inset figure indicates those processes included in the main figure). Also note that the cycling pool of vesicles has been omitted for clarity.
Redrawn from Michels *et al.* (2011).

5: From memory trace to conditioned behaviour

At the moment of testing, the odour-reward memory trace manifests itself in a preference for the previously rewarded over the previously non-rewarded odour (Fig. 1A, B). To see how the memory-trace-to-behaviour process is organized, we manipulated the test conditions such that the larvae were tested either in the absence or in the presence of the reward (Fig. 6A, B; Gerber and Hendel 2006, Schleyer et al. 2011). Strikingly, despite identical training and thus in possession of the same odour-reward memory trace, the presence of the training-reward prevents the larvae from showing appetitive conditioned behaviour (Fig. 6B, leftmost and middle group). This was shown to be the case both for sugar and for low-salt as reward. In turn, after aversive training, either with quinine or with high-salt, larvae express aversive conditioned behaviour in the presence of the punishment, but not in its absence (Gerber and Hendel 2006, Schleyer et al. 2011), and the same was found with respect to mechanosensory disturbance as punishment (Eschbach et al. 2011) (for a discussion of the generality of this effect see Schleyer et al. 2011). Importantly, tastants do not influence innate olfactory choice behaviour (Fig. 6C), and in turn odours do not affect innate gustatory behaviour (Fig. 6D). Thus, the gustatory situation specifically affects conditioned olfactory behaviour, while innate olfactory and innate gustatory behaviour seem to be mutually insulated.

These findings can be understood when conditioned behaviour, rather than as response to the odour, is regarded as an action in pursuit of its outcome (Dickinson 2001, Elsner and Hommel 2001, Hoffmann 2003, Gerber and Hendel 2006, Schleyer et al. 2011). Accordingly, appetitive conditioned behaviour is viewed as 'informed search' for food - which is abolished in the presence of the sought-for food. Conversely, aversive conditioned behaviour is viewed as 'informed escape' from punishment - which is disabled in the absence of punishment - much in the same way as we only search for things that are *not* present, and run for the emergency exit in a movie theater only when there *is* an emergency. Thus, conditioned behaviour is not an automatic response: Associative memory traces can be behaviourally expressed- or not.

Upon further analysis, it turned out that this organization of conditioned behaviour involves a comparative step: After training larvae with a particular concentration of sugar, they express conditioned behaviour only when the sugar concentration at the moment of testing is less than the

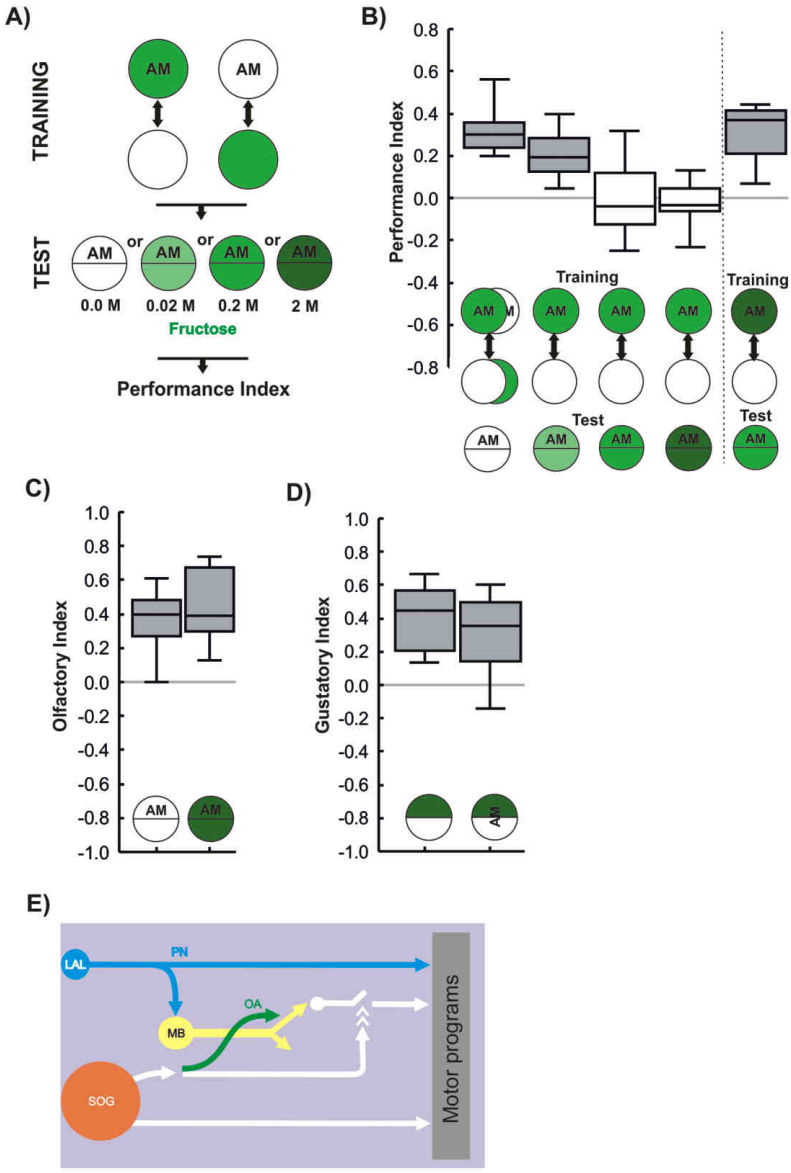

Figure 6

(A) Experimental design. Circles represent Petri dishes containing a sugar reward (fructose, green) or plain agarose (white). Larvae are trained such in the reciprocal groups *n*-amylacetate (AM) is either paired or not paired with a 0.2 M fructose reward. Subsequently, larvae are tested for their AM preference on either a tasteless, pure substrate, or in presence of 0.02 M, 0.2 M, or 2 M fructose. From the difference of olfactory behaviour of two reciprocally trained groups (tested on the same substrate), the Performance Index is calculated to quantify associative learning.

(B) Neither the training-concentration of fructose alone, nor the testing-concentration of fructose alone can account for conditioned behaviour. Rather, the *comparison* of both theses pieces of information determines whether conditioned behaviour is expressed. That is, appetitive conditioned behaviour is expressed only when the trained fructose concentration is *higher than* the fructose concentration in the moment of test.

(C) Innate, experimentally naïve olfactory preference toward AM is not influenced by the presence of fructose.

(D) Innate, experimentally naïve gustatory preference toward fructose is not influenced by the presence of AM.

(E) Circuit-model of the 'decision' process to behaviourally express an appetitive memory trace- or not. At the moment of test, the 'value' of the appetitive memory trace as read-out from the MB is compared to the 'value' of the currently detected appetitive gustatory inputs. The connection to express appetitive conditioned behaviour remains interrupted (arrowheads) as long as these gustatory inputs are at least as 'good' as predicted by the activated memory trace. Thus, the learned odour is tracked down only 'in search for more', i.e. when doing so promises a positive outcome or 'gain'. For the aversive case (not shown) (see Schleyer *et al.* 2011), the situation is inverse: Aversive memory traces are behaviourally expressed only when the testing situation is as 'bad' as or worse than the punishment used in training.

Other details as in Fig. 1.

(B-D) on the basis of data from Schleyer *et al.* (2011).

sugar concentration during training. Given that the larvae of the four leftmost groups displayed in Figure 6B had all been trained in the same way and thus will have established the same memory trace, it is obviously not the memory trace *per se* that determines the behaviour of the animals in the test. In turn, animals which are tested under one and the same sugar concentration but which have been trained with different concentrations also differ in behaviour: If the sugar concentration during training was higher than it is during testing, conditioned 'search for more' is expressed (Fig. 6B, rightmost group). Critically, at this very same test concentration of sugar, no conditioned behaviour is seen if the training concentration had been equally high as the testing-concentration (Fig. 6B, middle group). Thus, the testing situation *per se* is not a sufficient determinant of conditioned behaviour, either (this is in contrast to effects of visceral and/or metabolic feedback reported by Krashes et al. [2009] which arguably shuts down mushroom body output altogether at a site 'upstream' of the comparative process under study here). Rather, it is the comparison between the memory trace and the testing situation that needs to be considered (Fig. 6E): The larvae compare the value of the current situation (the 'is-state' based on gustatory input) to the value of the odour-activated memory trace (the 'could-get state' based on mushroom body output). Clearly, for this comparison to happen, the memory trace has to be 'read out', i.e. the mushroom bodies need to actually output - but off line from behaviour. The circuit toward conditioned search is only closed if the memory trace suggests a place better than the current one, in other words if there is something to search for, if there is a gain to be expected from tracking down the learned odour. It is this outcome expectation, and not the value of the memory trace, which is the immediate cause of conditioned behaviour.

This flexible, open organization of conditioned behaviour contrasts with the more rigid, closed processing stream mediating innate olfactory behaviour, which is expressed regardless of the testing situation (Fig. 6C). This separation of olfactory processing streams corresponds to the bifurcation of the projection neurons: The direct pathway toward the lateral horn mediates innate, closed kinds of olfactory tendencies, whereas the mushroom body detour corresponds to an open, flexible olfactory processing stream which can be regulated to express conditioned behaviour- or not.

6: Outlook

It seems that olfaction and olfactory learning in *Drosophila* are beginning to be understood satisfyingly well. Indeed, both in genuinely sensory aspects and in terms of olfactory memory trace formation reasonable working hypotheses seem within reach. These working hypotheses are detailed enough to make the gaps in understanding obvious, with respect to e.g. the exact organization of the aminergic system with respect to mnemonic processing, the processing of odour-intensity information and the way it is included in olfactory memories (Yarali et al. 2009), the change in valence of memory upon reversing stimulus timing during associative training (Tanimoto et al. 2004), the molecular and cellular architecture of gustation and the entanglement of gustation with mechanosensory processing, the role of visceral and/or metabolic feedback in associative processing, the molecular network organization of the presynapse, the role of the postsynapse in associative plasticity, the temporal dynamics of the memory trace(s) formed and their respective content, or the mechanisms of integration across sensory modalities.

It is precisely because the situation over-all is already fairly satisfying that an extension of scope is now called for. For example, the internally motivating factors of behaviour remain distressingly unclear. Likewise, one may ask whether, beyond the mere ‘good’ and ‘bad’, information about the particular kind of reinforcer and its intensity is contained in the memory trace, whether the animals ‘know’ when and how often they had been rewarded, or whether they experience degrees of ‘certainty’ about what they have learned. The combination of carefully designed and fine-grained behavioural analyses, together the cellular simplicity and genetic tractability of the *Drosophila* larva may allow to develop circuit-level accounts of such mnemonic richness. If it indeed exists.

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Chapter II:

Bitter processing in *Drosophila* larvae

II.1 Behavioural analyses of quinine processing in choice, feeding and learning of larval *Drosophila*

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Abstract

Gustatory stimuli can support both immediate reflexive behaviour, such as choice and feeding, and can drive internal reinforcement in associative learning. For larval *Drosophila*, we here provide a first systematic behavioural analysis of these functions with respect to quinine as a study case of a substance which humans report as "tasting bitter". We describe the dose-effect functions for these different kinds of behaviour and find that a half-maximal effect of quinine to suppress feeding needs substantially higher quinine concentrations (2.0 mM) than is the case for internal reinforcement (0.6 mM). Interestingly, in previous studies (Niewalda et al. 2008, Schipanski et al 2008) we had found the reverse for sodium chloride and fructose/sucrose, such that dose-effect functions for those tastants were shifted toward lower concentrations for feeding as compared to reinforcement, arguing that the differences in dose-effect function between these behaviours do not reflect artefacts of the types of assay used. The current results regarding quinine thus provide a starting point to investigate how the gustatory system is organized on the cellular and/ or molecular level to result in different behavioural tuning curves toward a bitter tastant.

Introduction

The sense of taste is that component of the contact chemosensory system devoted to organize feeding, allowing animals to prefer edible and avoid toxic substances. In addition, gustatory stimuli can be

reinforcers: They can induce memories for stimuli or actions that preceded them, such that the animal can find good and avoid bad food. Gustatory stimuli thus organize both immediate, reflexive behaviour toward food (such as choice and feeding), and, by virtue of their association with predictive stimuli or instrumental actions, the search for food. Trivially, these functions must come about by different sets of neurons on at least some level of processing. While at the level of gustatory interneurons such dissociation can clearly be found (e.g. in terms of the sufficiency of octopaminergic signalling for reinforcement, but not for ingestive behaviour [1-3]), it is not resolved in detail whether and how different sets of sensory neurons organize different gustatory reflex behaviours and/ or internal reinforcement signals, respectively (for an interesting study of this issue in mice see [4]). Here, we want to take a first systematic step into such an analysis by behaviourally "footprinting" the dose-effect characteristics of bitter-processing ("bitter" is used throughout this study in the sense that humans verbalize these chemically diverse and often toxic substances as "tasting bitter" and avoid eating them) in choice, feeding and reinforcement processing of larval *Drosophila*, using quinine as a study case.

The larva is the growth and feeding stage of the *Drosophila* life cycle and as such is a suitable study case for taste research. Substrate choice, feeding and reinforcement learning can be tackled by simple, cheap and well-defined behavioural assays; in addition, the larval gustatory system is comprised of relatively few neurons and is beginning to be described at the anatomical, cellular and to some extent also the molecular level [5-7] (for reviews with emphasis on the larva see [8-12]; for more general reviews on the neurogenetics of chemosensation see [13-18]). In principle, the cellular architecture of taste processing seems to conform to what had been found in adults (see reviews cited above) (Fig. 1). However, the exact relation between cellular identity, expression of putative gustatory receptor molecules from the *Gr*- [19] and/ or *Ir*-family of genes [20], their molecular mode of action, their ligand profile, the terminal projection patterns of their host gustatory sensory neurons and their behavioural roles are far from being satisfyingly clear (see Discussion). To take a first systematic step into an analysis of larval bitter-processing, we parametrically describe at the behavioural level the effects of various concentrations of quinine hemisulfate. Specifically, we examine the following questions:

- How does quinine concentration affect choice between bitter and tasteless substrates?
- How does quinine concentration affect feeding behaviour?
- How do different quinine concentrations differ in their reinforcing power?
- How do the respective dose-effect curves relate?

These experiments, we hope, will provide a framework to investigate how the gustatory system is "orchestrated" on the cellular and/ or molecular level to support different kinds of behaviour toward bitter tastants. Such a study case of the gustatory system of larval insects is interesting also from an applied perspective as these creatures eat up sizeable fractions of the global agricultural harvest.

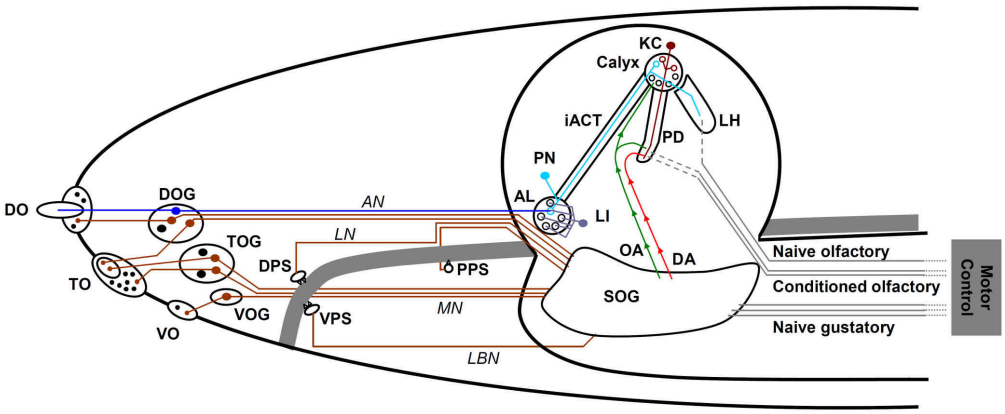

Figure 1: The anatomy of the *Drosophila* chemosensory system
Overview of the cephalic larval chemosensory pathways. Olfactory pathways (blue) project into the brain *proper*, whereas gustatory afferents (brown) are collected in various regions of the subesophageal ganglion. The green and red arrows indicate pathways to short-circuit a taste-driven reinforcement signal from the subesophageal ganglion toward the brain. Note that the different gustatory organs project to different regions in the subesophageal ganglion.
Abbreviations: AL: antennal lobe; AN: antennal nerve; DA: dopaminergic neurons as engaged in reinforcement signalling; DO/ DOG: dorsal organ/ dorsal organ ganglion; DPS: dorsal pharyngeal sensillae; iACT: inner antenno-cerebral tract; IN: antennal lobe interneurons; KC: Kenyon cells; LBN: labial nerve; LH: lateral horn; LN: labral nerve; MN: maxillary nerve; OA: octopaminergic neurons as engaged in reinforcement signalling; PN: projection neurons; PPS: posterior pharyngeal sensillae; SEG: subesophageal ganglion; TO/ TOG: terminal organ/ terminal organ ganglion; VO/ VOG: ventral organ/ganglion; VPS: ventral pharyngeal sensillae.
Based on Stocker 2008 [10].

Materials and methods

Larvae

We use third-instar feeding-stage larvae from the Canton-Special wild-type strain, aged 5 days after egg laying. Flies are maintained on standard medium, in mass culture at 25 °C, 60- 70 % relative humidity and a 14/ 10 hour light/ dark cycle. Before each experiment, we remove a spoonful of food

medium from a food vial, collect the desired number of larvae, briefly rinse them in distilled water and start the experiment.

Choice

The day before experiments, we prepare the Petri dishes (with 55 mm inner diameter; Sarstedt, Nümbrecht, Germany). We separate them into two halves with a piece of overhead transparency, fill one side with only 1 % agarose (henceforth called PURE; electrophoresis grade; Roth, Karlsruhe, Germany) and the other side with 1 % agarose added with quinine hemisulfate as a bitter tastant (henceforth called QUI; CAS: 6119-70-6; purity: 94 %, Sigma-Aldrich, Seelze, Germany) at the respectively indicated concentrations; for the CONTROL condition, both sides of the Petri dish contain PURE. Shortly before the agarose is completely solidified, we remove the overhead transparency, and after appr. 10 minutes of cooling we cover the dishes with their lids and leave them at room temperature until the following day. Please note that we cannot exclude some level of diffusion of quinine into the PURE half of the Petri dish overnight, such that there might be a quinine gradient in the middle section of the Petri dish. Therefore we determined an appr. 1 cm wide middle zoe. Larvae inside this middle zone are included in the total number of equation (1) (see below). This procedure may underestimate QUI avoidance, but cannot lead to false-positive results.

Unless mentioned otherwise, we place 15 larvae in the middle of the dish and close the lid. The QUI-side is in half of the cases to the right and in the other half to the left, to balance for spurious effects of the experimental surround. We record the number of larvae on either side of the dish and calculate a gustatory preference index ($PREF_{Gustatory}$) as:

$$(1) \qquad PREF_{Gustatory} = \frac{\#_{QUI} - \#_{PURE}}{\#_{TOTAL}}$$

In this equation, # indicates the number of larvae on the respective side of the dish. Thus, $PREF_{Gustatory}$ values are constrained between 1 and -1, positive values indicating a preference for QUI

and negative values indicating aversion. These scores are taken at various time points after the animals are placed onto the dish (see Results for details).

Feeding

To measure feeding behaviour on substrates containing QUI, we follow a procedure based on [21]: Ten larvae are placed on a 90 mm diameter Petri dish filled with either 1 % agarose plus 30 % red food dye (RU9805; http://www.backfun.de) (these are henceforth called PURE) or are filled with agarose, the food dye, plus the chosen concentration of QUI (see Results for details). The animals are allowed to feed on either of these respective substrates for 15 min; then they are washed in tap water, transferred onto a Petri dish and placed on crushed ice for approximately 3 min. Next, a haphazardly chosen individual larva from the PURE Petri dish is taken, briefly (approximately 2 s) put into boiling water and placed under a binocular coupled to a digital camera (Canon, model A650); we then place a haphazardly chosen companion larva from the QUI Petri dish next to the larva from the PURE dish and take a picture. For illumination we use a light table coupled to a cold-light source (Volpi, Schlieren, Switzerland). Using an imageJ-based, custom-written software, we determine for each larva the area of its body (Body) and the area of red colour (Red) in its gut. From these data, we calculate a Feeding Index (FI) as:

$$\text{FI} = \frac{\text{Red}_{\text{QUI}}}{\text{Body}_{\text{QUI}}} - \frac{\text{Red}_{\text{PURE}}}{\text{Body}_{\text{PURE}}} \quad (2)$$

In this equation, Red_{QUI} indicates the area of red colour of an individual larva fed on QUI, and Body_{QUI} indicates the area of its body. Red_{PURE} indicates the area of red colour and $\text{Body}_{\text{PURE}}$ the area of the body of the concomitantly photographed larva fed on PURE. Because the body area cannot possibly be smaller than the red-coloured area, FI values range from -1 to +1, with negative values indicating suppression of feeding by quinine and positive values indicating enhancement of feeding. Total body size did not differ between experimental groups (data not shown).

Reinforcement

These experiments use Petri dishes of 90 mm diameter filled with either only 1 % agarose (PURE) or with 1 % agarose added with quinine (QUI) as negative reinforcer (-) at the concentrations indicated along the Results section.

Prior to experiments, odour containers are prepared: 10 µl of odour substance is filled into custom-made Teflon containers (5 mm inner diameter with a lid perforated with seven 0.5-mm diameter holes). As odour, we use *n*-amyl acetate (AM, 99 % purity; Merk, Hohenbrunn, Germany), diluted 1:50 in paraffin oil (Merk, Darmstadt, Hohenbrunn); in cases when no odour is presented, an empty container is used (EM) because the paraffin solvent is behaviourally ineffective for the larvae [21,22]. Before the experiment starts, Petri dishes are covered with modified lids perforated in the centre by 15 holes with 1 mm diameter to improve aeration.

For training, we use a modified version of the one-odour reciprocal training regimen [22]. Thirty larvae are placed in the middle of a reinforcer-added dish with two odour containers on opposite sides (7 mm from the edges), both filled with AM. After 5 min, larvae are transferred onto an agarose-only dish with two empty containers, where they also spend 5 min. Three of these AM-/ EM training cycles are performed, each using fresh dishes. Along repetitions of the experiment, in half of the cases training starts with a reinforcer–added dish (AM-/ EM for all three training cycles) and in the other half with an agarose-only dish (EM/ AM- for all three training cycles).

Once training is completed, larvae are transferred to the middle of a quinine-containing Petri dish with two odour containers, this time filled with AM on one side and empty on the opposite side, to create a choice situation. Quinine is required during testing because aversive conditioned behaviour toward odour is a conditioned escape behaviour that is behaviourally expressed only if the test situation does indeed warrant escape (for a discussion see [23,24]). After 3 min, the number of larvae on each side of the dish is noted and an olfactory preference (PREF) is calculated as:

$$\text{PREF} = \frac{\#_{\text{AM}} - \#_{\text{EM}}}{\#_{\text{TOTAL}}} \quad (3)$$

In this equation, # indicates the number of larvae observed on the respective side of the dish. PREF values thus can range between 1 and -1, positive values indicating preference for and negative values avoidance of AM.

For each group of larvae trained AM-/ EM (or EM/ AM-, respectively), a second group is trained reciprocally, i.e. by unpaired presentations of odour and quinine: AM/ EM- (or EM-/ AM, respectively). Aversive associative learning shall result in a stronger avoidance for AM after AM-/ EM training than after AM/ EM- training. This difference is quantified by the performance index (PI) as:

$$\text{PI} = \frac{\text{PREF}_{\text{AM-/ EM}} - \text{PREF}_{\text{AM/ EM-}}}{2} \quad (4)$$

Here, $\text{PREF}_{\text{AM-/ EM}}$ is the AM preference of the AM-/ EM trained group and $\text{PREF}_{\text{AM/ EM-}}$ is that of the reciprocally trained AM/ EM- group (or of the respectively other training trial sequence). This PI is a measure of associative learning because it measures the difference in preference between two groups trained reciprocally, but otherwise treated the same (i.e. with respect to handling, exposure to odours and exposure to the reinforcer). PI values thus range between 1 and -1, positive values indicating conditioned approach toward the reinforcer-paired odour (appetitive learning) and negative values indicating conditioned avoidance of the reinforced odour (aversive learning).

Statistical analyses

All statistical analyses are performed with Statistica on a PC. Preference values, feeding indices and performance indices are compared across multiple groups with Kruskal-Wallis tests. For subsequent pair-wise comparisons, Mann-Whitney U-tests are used. To test whether values of a given group differ from zero, we use one-sample sign tests. When multiple one-sample sign tests, Kruskal-Wallis tests, or Mann-Whitney U-tests are performed within one experiment, we adjust significance levels by a Bonferroni correction to keep the experiment-wide error rate at 5 %. This is done by dividing the critical *P* value of 0.05 by the number of tests. We present our data as box plots which represent the

median as the middle line and 25% / 75% and 10 % / 90 % as box boundaries and whiskers, respectively.

Results

Choice

First, we seek a suitable assay duration for testing bitter avoidance of experimentally naïve larvae; this is warranted because here, following the approach of Schipanski and colleagues analyzing sugar-initiated behaviour [25] we use assay plates with smaller diameter (approximately 55 mm) than in previous studies on quinine-related behaviour (approximately 90 mm) [21]. We restrict ourselves to a total observation time of 8 min because after that time point the larvae begin to dig into the substrate or to crawl up the side walls of the Petri dish (not shown); we chose 5 mM of QUI because this concentration had been used in previous work and because higher concentrations of QUI, without acidification, show crystalization of QUI in the agarose dishes. Thus, we allow the larvae to choose between pure agarose (PURE) and agarose added with 5 mM QUI and recurrently score for their choice after 1, 2, 4, and 8 min.

We observe avoidance of 5 mM QUI beginning already from 1 min after assay-onset (Fig. 2A; one-sample sign tests $P< 0.05/ 4$ for all time points; sample size N= 101). We chose 8 min as observation time for all subsequent analyses, as for this time point avoidance was apparently strongest, albeit not yet asymptotic. We note that we have previously found that when using fructose, sucrose or trehalose rather than QUI, asymptotic preference is found after less than 8 min [25], arguing that from the motor side and the spatial layout of the assay the allowed time is enough for the larvae to behaviourally express their preference, if they have any.

We next asked whether, when assayed after 8 min, the avoidance of QUI depends on its concentration. Using either CONTROL Petri dishes that contain PURE agarose on both sides or Petri dishes with one side including QUI and the other side PURE, we probe for QUI avoidance in a range from 5 mM down to 0.005 mM. Obviously, the concentration of QUI matters for larval avoidance behaviour (Fig. 2B; Kruskal-Wallis test: $P< 0.05$, H= 150, df= 4, sample sizes N= 110, 150, 120, 95, 101). When both sides of the Petri dish lack QUI, the larvae distribute equally between both sides (Fig.

2B; CONTROL one-sample sign test: P= 0.36; sample size as above). However, already for the lowest concentration (0.005 mM) larvae show a weak yet, given the very large sample size, significant avoidance of QUI (Fig. 2B; one-sample sign tests: P<0.05/ 5; sample size as above) (for the respective other concentrations: P< 0.05/ 5 as well; sample sizes as above). Notably, values for the two highest concentrations still do differ from each other (Fig. 2B; Mann-Whitney U-test; U= 2969; P< 0.05; sample sizes as above), arguing that the point of asymptote should be above 0.5 mM, and thus beyond the range of concentrations that can be used without acidifying the agarose (see above).

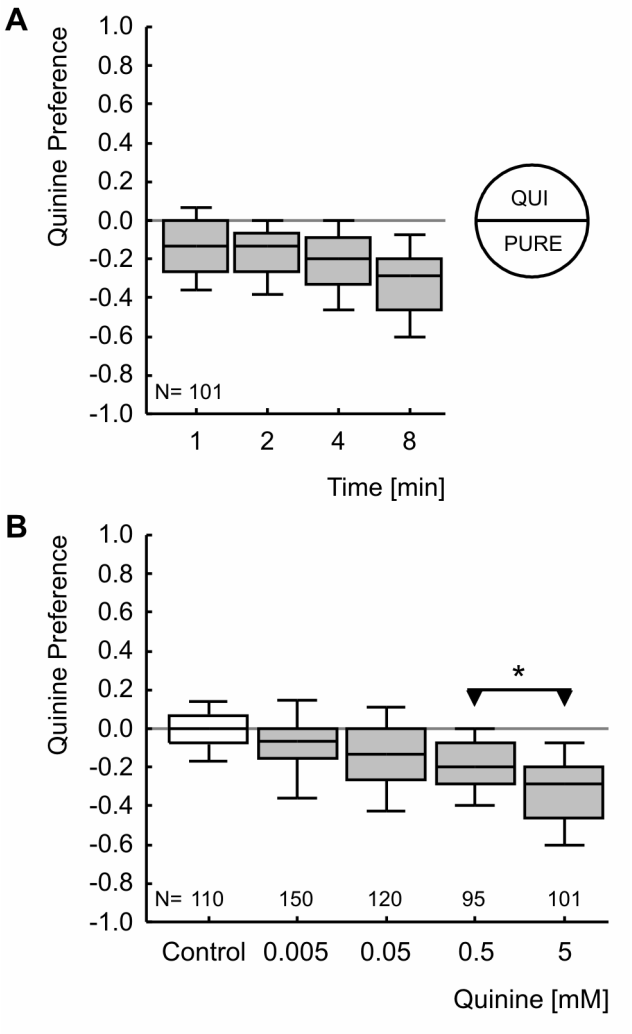

Figure 2: Choice
(A) Larvae are allowed to chose between one side of a Petri dish that contains agarose added with 5 mM quinine (QUI), and pure agarose (PURE) on the other side. A gustatory preference ($PREF_{Gustatory}$) is calculated at different time points after the experiment has started. Negative $PREF_{Gustatory}$ values indicate avoidance of QUI which is statistically significant already from 1 min on. Based on these results we chose 8 min as time point of scoring choice in subsequent experiments, as for this time point avoidance was apparently strongest.
(B) Gustatory preference either between both sides of a Petri dish filled with agarose-only (CONTROL) or between a QUI- and a PURE-filled side of a split Petri dish, at the indicated concentrations of QUI. Scores are taken after 8 min. At concentrations from 0.005 mM to 5 mM QUI is avoided by the larvae. Avoidance differs between the two highest concentrations of QUI, thus arguing that the point of asymptote should be above 5 mM.
Shading of the boxes indicates significant differences from chance behaviour (i.e. zero, one-sample sign tests) (A) P< 0.05/ 4; (B) P< 0.05/ 7; keeping the experiment-wide error rate at 5 % (i.e. Bonferroni correction). Labelling of * refers to P< 0.05 in a Mann-Whitney U-test. Box plots represent the median as the middle line and 25 %/ 75 % and 10 %/ 90 % as box boundaries and whiskers, respectively. Sample sizes: (A) N= 101; (B) from left to right N= 110, 150, 120, 95, 101.

Feeding

In the next experiment we seek to confirm the previously reported suppressing effect of 5 mM QUI on larval feeding [21] (Fig. 3A) and to extend that finding with respect to its dose-effect characteristic (Fig. 3B; Kruskal-Wallis test: $P< 0.05$, H= 75.2, df= 6, sample sizes N= 90, 70, 200, 119, 147, 70, 162). Concentrations of QUI ranging from 0.5 mM to 5 mM lead to feeding suppression (Fig. 3B; one-sample-sign tests: $P< 0.05/ 7$, sample sizes see above); the two lowest QUI concentrations (0.05 mM and 0.16 mM), however, leave feeding unaffected (Fig. 3B; one-sample-sign tests: $P> 0.05/ 7$, sample sizes see above). Feeding Indices do not differ between the two highest concentrations (Fig. 3B; Mann-Whitney U-test: $P> 0.05/ 2$, U= 5017, sample sizes as above), arguing that the asymptote of feeding suppression is reached at a concentration of lower than 3 mM; as, in turn, feeding suppression for 3 mM is stronger than for the next lower concentration (Fig. 3B; Mann-Whitney U-test: $P< 0.05/ 2$, U= 3825, sample sizes as above), the concentration of asymptote should be higher than 1.6 mM.

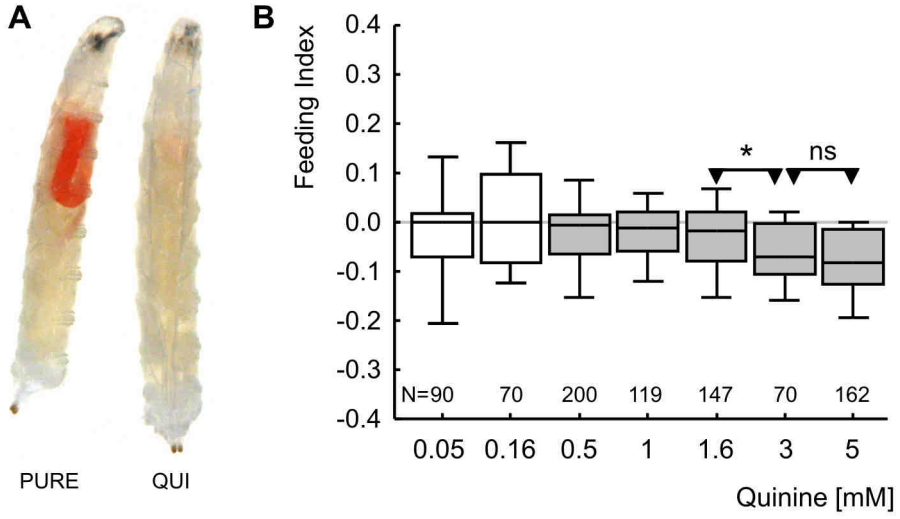

Figure 3: Feeding
(A) Larvae are allowed to feed on either a red-dyed, PURE Petri dish (left) or on a red-dyed, 5 mM QUI-containing Petri dish (right), for 15 minutes. Using a digital camera, a picture is taken and the area of red colour in individual larvae (indicating the amount eaten) as well as the area size of the whole body is determined for the calculation of the Feeding Index.
(B) Feeding Indices of larvae fed on QUI in a concentration range between 0.05 mM and 5 mM. Positive Feeding Index values (FI) indicate that larvae eat more on a QUI than on a PURE Petri dish, negative scores indicate QUI-induced suppression of feeding. Larvae show feeding suppression for QUI concentrations in a range from 0.5 mM to 5 mM. Please mind the truncated axis.
Shading of the boxes indicates significant differences from chance behaviour (i.e. zero, one-sample sign tests) ($P< 0.05/ 7$, keeping the experiment-wide error rate at 5 % [i.e. Bonferroni correction]); labelling of * or ns refers to $P< 0.05/ 2$ or $P> 0.05/ 2$ in Mann-Whitney U-tests. Box plots represent the median as the middle line and 25 %/ 75 % and 10 %/ 90 % as box boundaries and whiskers, respectively. Sample sizes: from left to right N= 90, 70, 200, 119, 147, 70, 162.

Reinforcement

Next, we probe QUI-induced aversive learning for its dose-dependency. We train larvae with either of six concentrations of QUI (5 mM, 1.61 mM, 0.5 mM, 0.16 mM, 0.05 mM, 0.005 mM) and compare these concentrations in terms of their reinforcement potency (Fig.4; for the corresponding preference scores see Fig. S1). These different QUI concentrations differ in terms of the associative Performance Index they support (Fig. 4; Kruskal-Wallis test: $P< 0.05$, H= 56.5, df= 5, sample sizes N= 13, 46, 29, 49, 29, 76), such that the three highest concentrations (5 mM, 1.61 mM and 0.5 mM) support significant aversive learning (Fig. 4; one-sample sign-tests: $P< 0.05/ 6$, sample sizes as above), whereas the three lowest concentrations (0.16 mM, 0.05 mM and 0.005 mM) do not (Fig. 4; one-sample sign-tests: $P> 0.05/ 6$, sample size as above). When compared between the two highest concentrations, Performance Indices do not differ (Fig. 4; Mann-Whitney U-test: $P> 0.05/ 2$, U= 988, sample sizes as above), arguing that the reinforcement potency of QUI reaches asymptote at a concentration lower than 1.61 mM; as, in turn, Performance Indices for the second-highest concentration are stronger than for the middle concentration (Fig. 4; Mann-Whitney U-test: $P< 0.05/ 2$, U= 434, sample sizes as above), the point of asymptote should be higher than 0.50 mM.

Comparing dose-effect functions for feeding and reinforcement

Given that for feeding and for the reinforcement function the concentration at which QUI exerts an asymptotic effect, and thus the concentration at which the effect is half-maximal, could be determined (Fig.s 3,4), we decided to provide an overview of the these results as follows: We divide the median Feeding Index for each concentration by the strongest median Feeding Index found (i.e. by the one for 5 mM QUI) (and multiply by -1), thus yielding the normalized Feeding scores displayed in Figure 5 (red). The data from the leaning experiment (Fig. 4) were treated accordingly, yielding the normalized Learning scores displayed in Figure 5 (green).

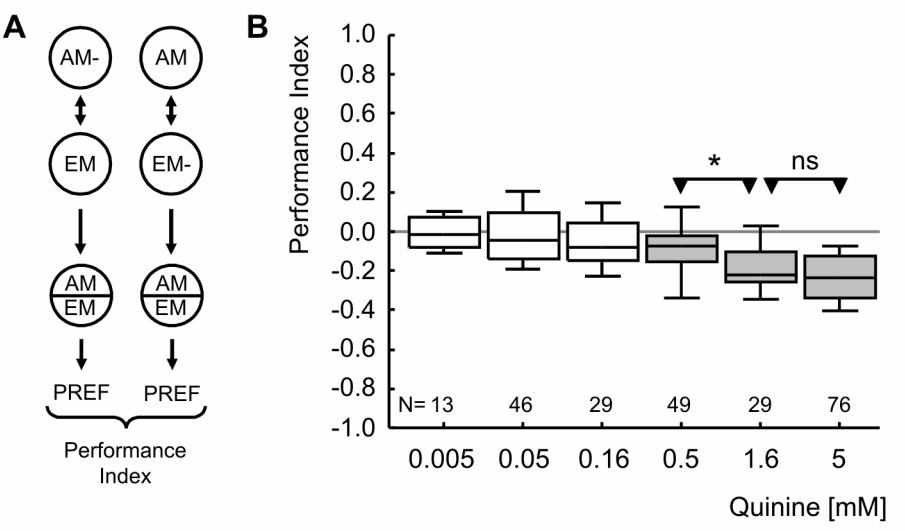

Figure 4: Reinforcement
(A) Larvae are trained such that one group of larvae receives *n*-amylacetate while crawling on a QUI-containing Petri dish, whereas an empty odour container is presented in the absence of the reinforcer (i.e., AM-/EM training; left). Another group is trained reciprocally, i.e. with unpaired presentations of AM and reinforcement (AM/ EM-; right) (note that for half of the cases the sequence of trials is as indicated; for the other half, sequences are reversed: EM/ AM- and EM-/ AM, respectively). After three such training cycles, larvae from both groups are tested on a QUI-containing Petri dish for their preference between AM and EM in a choice situation (for a documentation of these preference scores, see Figure S1). Associative learning is revealed by lower preference scores for AM in the group trained AM-/ EM than in the reciprocally trained AM/ EM- group. This difference is quantified by the displayed Performance Index (PI), such that negative PI values indicate conditioned avoidance.
(B) The strength of QUI reinforcement depends on its concentration. In the range of concentrations tested (from 0.005 mM to 5 mM) the three highest concentrations support learning, but the three lowest concentrations do not. We find that QUI reinforcement reaches asymptote between 0.5 mM and 1.61 mM.
The shading of the boxes indicates significant differences from zero, i.e. from chance behaviour ($P< 0.05/ 6$ in one-sample sign tests, keeping the experiment-wide error rate at 5 % [i.e. Bonferroni correction]); labelling of * or ns refer to $P< 0.05/ 2$ or $P> 0.05/ 2$ in Mann-Whitney U-tests. Box plots represent the median as the middle line and 25 %/ 75 % and 10 %/ 90 % as box boundaries and whiskers, respectively. Sample sizes are from left to right N= 13, 46, 29, 49, 29, 76.

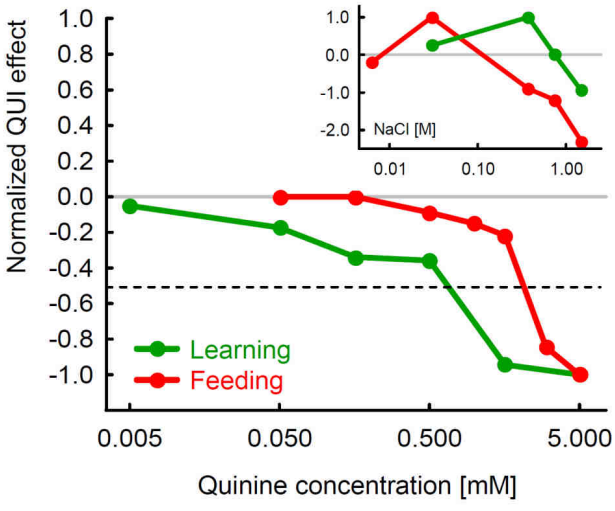

Figure 5: Summary of the dose-effect functions of feeding and learning
We divide the median values for each quinine concentration by the lowest median value found within one experiment (and multiply by -1), thus yielding the displayed normalized scores (green: Learning; red: Feeding). The half-maximal effect of QUI on feeding appears to require a concentration of QUI that is almost one order of magnitude higher than for learning. As for choice the maximal effect can for practical reasons not be determined (see Discussion), a similar display for choice would insinuate a too low concentration as "half-maximal" (see Fig. S2), and is therefore not included. The inset uses data from [26] to display the dose-effect functions of feeding and learning for sodium chloride for comparison. Please note that changing the concentration of sodium chloride in the rearing-food may alter the balance between its appetitive and aversive effects [36].

Discussion

In the present experiments on feeding and learning the concentrations at which the behavioural effects of quinine reach asymptote, and hence those concentrations which yield half-maximal effects, could be determined (Fig. 5); for choice, however, such asymptote is not reached at the highest concentration used in the current experiments (Fig. S2). As mentioned, that concentration (5 mM QUI) is the highest one that can be used without acidification; however, such acidification is not warranted because it introduces confounding gustatory and olfactory cues. In this context, one may wonder what the acidity of those microhabitats is in which the larvae encounter QUI in the wild, and/or whether the more natural role of QUI in choice behaviour is a modulation of sugar processing (see below). Still, from a practical point of view, another option to detect the asymptote of QUI choice behaviour would have been to allow the larvae more time for choice; however, as mentioned above we observed that for longer assay durations the larvae start digging into the agarose. Thus, with the present experiments we cannot determine the "true" asymptote of quinine choice behaviour. Therefore we restrict the below discussion to feeding and learning.

Comparing the dose-effect functions for feeding and learning

For feeding, a half-maximal effect appears to require a concentration of QUI that is substantially higher than for learning (Fig. 5). Why is this so?

Potentially, these differences could be due to differences in sensitivity of the respective behavioural assays: For example, feeding as the primary occupation of the larvae may be particularly hard to suppress. Also, the learning assay measures the behaviour of populations of larvae, while the feeding assay considers individual behaviour, potentially making decreases in feeding more difficult to detect. However, when using NaCl rather than QUI, Niewalda and colleagues [26] found that the dose-effect function for feeding modulation is shifted by one order of magnitude toward lower concentrations as compared to learning (see inset of Fig. 5), and Schipanski and colleagues [25] reached the same conclusion regarding fructose/sucrose, lending no support to the notion that the feeding assay *per se* were, for whatever reason, different in sensitivity from the learning experiment.

As an alternative explanation, feeding and the reinforcing effect of QUI may rely on distinct processing streams, differing in their dose-effect characteristics (Fig. 6):

- Suppose different sets of receptor molecules (R-1 and R-2) were expressed in different sensory neurons (SN-1 and SN-2), differing in their dose-effect characteristics (indicated by the thickness of the lines). Thus, the behaviours respectively steered by them will follow these respective tunings (Fig. 6A).
- Suppose pharyngeal sensory neurons were involved in organizing feeding behaviour but externally located sensory neurons were responsible for triggering reinforcement (Fig. 6B). As the ingested substrate will be diluted by saliva, the actual concentration of QUI in the pharynx will be lower at the internal taste organs, requiring the experimenter to use higher concentrations to reach full effect for feeding. While in principle conceivable, we note that regarding sodium chloride and fructose/sucrose the argument would need to be made just the other way around (see above).
- Lastly, a given sensory neuron may form connections toward downstream neurons at different gains. For example, downstream neurons suppressing feeding may require stronger input from sensory neurons than neurons mediating internal reinforcement (Fig. 6C).

In principle, the relatively fragmented knowledge of the larval contact chemosensory system allows for either of these three interpretations: Candidate larval gustatory sensory neurons are located in both external and internal sense organs (Fig. 1), both of which include non-gustatory sensory neurons as well (whether the so-called p-es organs in the ventral pits [12] on the thoracic and/or abdominal segments contribute to processing of classical tastants is presently unknown). The external ones are the terminal (26 gustatory sensory neurons) and the ventral organ (7) and the bulge of the dorsal organ (9). The internal sensillae are located along the pharynx and are organized into dorsal, ventral and posterior pharyngeal sensillae (16; 15; 6) [6,7]. All these gustatory sensory neurons project to the subesophageal ganglion (SEG) (Fig. 1) [6,7]. Importantly, in larval and in adult *Drosophila*, target regions of gustatory sensory neurons within the SEG seem to depend both on the location of the peripheral taste organs from which they originate and on their behavioural function [6,7,18].

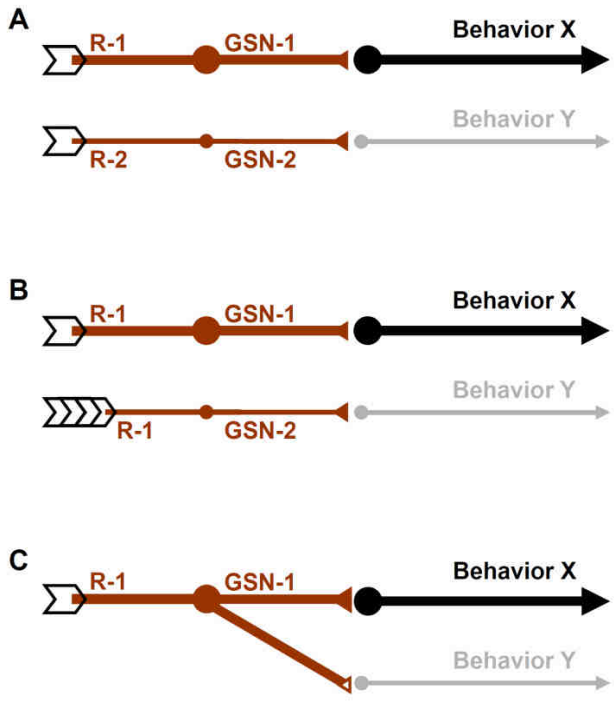

Figure 6: Accounting for differences in dose-effect functions for different behaviours
Schematic sketches of how different dose-effect characteristics of bitter processing with respect to different behaviours could come about. (A) Different sensory neurons (GSN-1, GSN-2) steering different behaviours (Behaviour X and Behaviour Y) may differ in the receptors expressed (R-1, R-2, with different ligand sensitivity), or (B) in the sensory-organ origin of their host sensory neurons, such that one of them is less exposed to QUI because of e.g. dilution by saliva. (C) One and the same subset of sensory neurons may diverge to form synaptic connections of different gain to downstream neurons. In all three cases, a particular QUI concentration would be more powerful to elicit behaviour X than behaviour Y.

On the molecular level, primary bitter as well as sugar taste processing in larval and adult *Drosophila* at least partially relies on the *Gr*- and possibly the *Ir*- [18] and/or *Trp*-family [27] of receptors: In adult *Drosophila*, distinct sets of gustatory sensory neurons express putative sugar (*Gr5a*) or bitter (*Gr66a*) receptor genes [28,29]. Driving *Gr5a*- or *Gr66a*-positive neurons is sufficient to induce attraction or avoidance, respectively [29]. However, bitter tastants can not only activate the neurons that express a respectively tuned receptor, but can also inhibit sugar receptor-expressing cells as well as water-sensitive cells when presented in mixture with sugars or in aqueous solution [30]. Consistent with this triple effect of bitter tastants, disabling the *Gr66a*-positive neurons reduces, but does not abolish, the capacity of bitter tastants to inhibit proboscis extension toward sugars [28]. Notably, bitter-sensitive gustatory neurons typically seem to express more than one *Gr* gene [17,31-34].

Regarding the larva, *Gr66a* is expressed in the larval terminal organ and likely also in gustatory neurons along the pharynx [7,35]. Like in adults, all gustatory cells of the terminal organ expressing *Gr66a* also express *Gr33a*. In addition, 15 other *Grs*, including *Gr32a*, are co-expressed with *Gr66a* in partially overlapping sets of cells [35]. Also, driving *Gr66a*-expressing neurons induces larval

avoidance behaviour [7], but strikingly no sugar-sensitive *Gr* has yet been found in larvae [35]. A similarly detailed analysis of *Irs* in the larva is not yet available.

In adults it has been reported that there exist different types of taste sensillae responding to different subsets of bitter substances [30,34]. For example, after genetic ablation of the TrpA1 channel, behaviour of adults toward aristolochic acid was reduced, without affecting behaviour regarding other bitter substances like quinine, denatonium, berberine or strychinine [27].

To summarize, it appears reasonable to reckon with substantial diversity between bitter-sensitive sensory neurons, including differences in their receptor complements, ligand profiles, dose-effect characteristics, connectivity to downstream neurons and behavioural roles. It should be interesting to see whether this diversity will eventually be seen as drastic enough to abandon the bitter-category altogether. In any event, for quinine as an example this study provides a first systematic step to investigate how the gustatory system in the larva is organized to support different kinds of behaviour toward a bitter tastant.

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Supplement

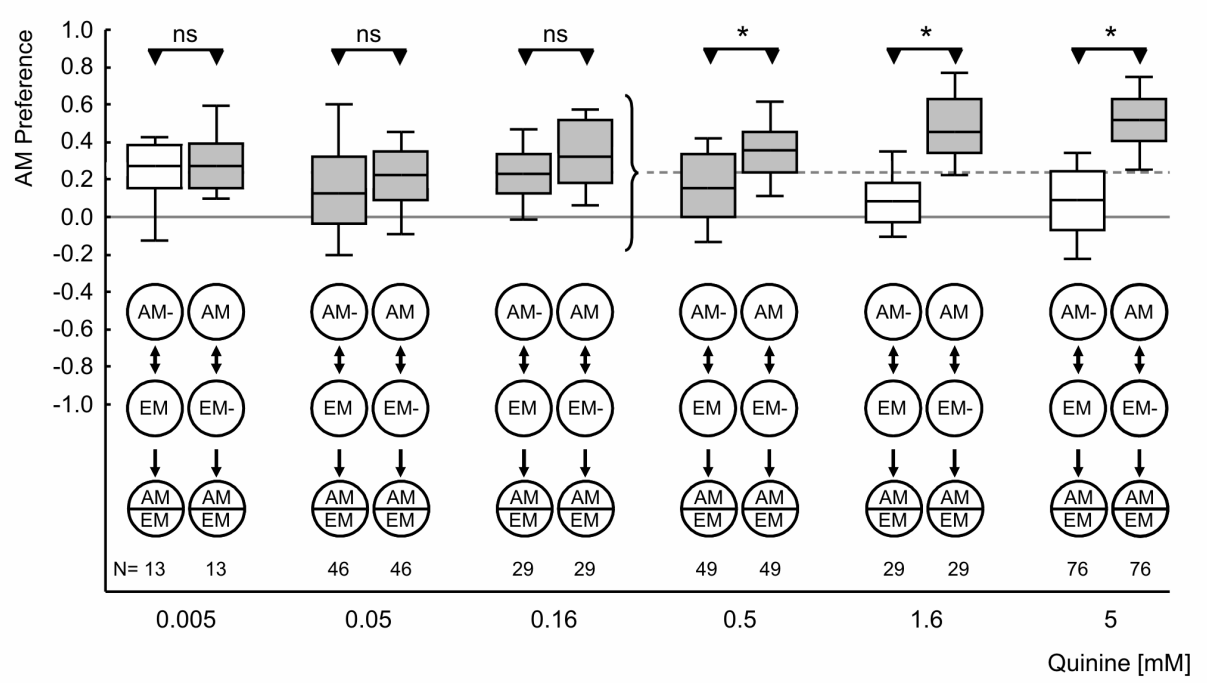

Figure S1: Preference scores of all groups of larvae from the reinforcement experiment
Animals receive either an AM-/ EM training or an AM/ EM- training and are subsequently tested on a QUI-containing Petri dish for their choice between AM and EM, as indicated in the sketches below the boxes. Differences in preference scores between two corresponding reciprocally trained groups (e.g. the two right-most panels) result in Performance Index (PI) scores different from zero (see Fig. 4, right-most panel). Note that in half of the cases the sequence of training trials is as indicated (in the right-most panel e.g. AM/ EM-), but in the other half it is reversed (e.g. EM-/ AM). The stippled line represents the median of the pooled six left-most boxes, showing that for higher concentrations of QUI paired and unpaired presentations of AM and QUI result in decreases and increases in preferences scores, respectively. The shading of the boxes indicates significant differences from zero in one-sample sign tests, i.e. from chance behaviour ($P< 0.05/ 12$, keeping the experiment-wide error rate at 5 % [i.e. Bonferroni correction]); labelling of * or NS refer to $P< 0.05/ 6$ or $P> 0.05/ 6$ in Mann-Whitney U-tests. Box plots represent the median as the middle line and 25 %/ 75 % and 10 %/ 90 % as box boundaries and whiskers, respectively. Sample sizes are from left to right N= 13, 13, 46, 46, 29, 29, 49, 49, 29, 29, 76, 76.

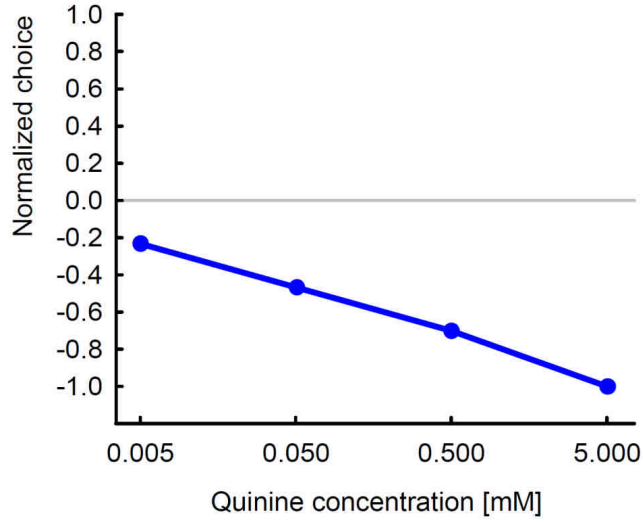

Figure S2: Dose-effect function of choice behaviour
We divide the median choice values for each quinine concentration by the lowest median value found (and multiply by -1), thus yielding the displayed normalized choice scores. The dose-effect curve seems to be linear, because for practical reasons a plateau in choice scores cannot be determined (see Discussion).

II.2 Interaction between bitter and sweet processing in larval *Drosophila*

Manuscript, in preparation

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Abstract

Gustatory stimuli play a crucial role in analysing the environment and discerning, for example, edible from toxic substances. In *Drosophila* larvae, 'sweet-' and 'bitter-tasting' substances can support attractive and aversive choice behaviour, respectively. We find that in addition the preference toward fructose is inhibited when assayed in the background of the bitter-tasting substance quinine, in a concentration dependent manner. Importantly, when testing the influence of quinine on the preference to various sweet tastants, we find that they differ in their susceptibility to be inhibited by quinine. Furthermore, the attractiveness of the sweet tastants is dissociated from their susceptibility to be inhibited by quinine. We discuss alternative functional models of how this effect may come about, and how these models can be tested experimentally.

Introduction

Discriminating edible from inedible substances is a critical task for any animal to survive. It is mainly managed by the animal's gustatory system: Sweet taste hints to nutritional, energy-rich food, whereas bitter taste is associated with toxic compounds in many cases. (Please note that in this study we refer to tastants as being 'sweet' or 'bitter', respectively, based on whether humans would classify them as such.) Therefore, sweet tastants are usually attractive, enhance feeding and act as appetitive reinforcers in associative learning. Bitter tastants, in turn, usually evoke aversion, suppress feeding and act as aversive reinforcers. Investigating the gustatory system of insects and especially insect larvae is of particular interest not only due to its implications to more complex behaviours such as learning or

decision-making (Schleyer et al. in press), but also from an ecological point of view: Insect larvae regularly cause huge damage to crops worldwide. To know how their sense of taste, their appetite and their choice behaviour are regulated could be of enormous benefit for agriculture.

The fruit fly *Drosophila melanogaster* is widely used as model organism for neurobiological studies. In the last decade, also *Drosophila* larvae received increasing experimental attention. They combine a numerically simple brain (roughly 10'000 neurons), correspondingly moderate behavioural complexity and *Drosophila*'s general potential for transgenic manipulation (Sokolowski 2001; Elliott and Brand 2008). Together, these features make them an attractive study case.

Our knowledge about the larval gustatory system is rather fragmentary (for detailed reviews on the *Drosophila* gustatory system, see Gerber and Stocker 2007; Cobb et al. 2009; Montell 2009; Tanimura et al. 2009). Taste information is sensed *via* appr. 90 gustatory sensory neurons per body side (Colomb et al. 2007), distributed across three external (terminal organ, ventral organ and the bulge of the dorsal organ) and three internal (ventral, dorsal and posterior pharyngeal) taste sensilla (Singh and Singh 1984; Python and Stocker 2002; Gendre et al. 2004). All these gustatory sensory neurons project to various regions of the suboesophageal ganglion (SOG), depending on both the sense organ they originate from and the receptor genes they express (Colomb et al. 2007). Knowledge of the connections from the SOG toward the brain (Melcher and Pankratz 2005; Colomb et al. 2007; Selcho et al. 2009) as well as toward (pre-)motor neurons presumably in the ventral nerve cord in particular remains fragmentary.

On the molecular level, sweet and bitter tastants likely are detected by G-protein coupled receptors of the *Gr* gene family (Clyne et al. 2000; Dahanukar et al. 2001; Thorne et al. 2004; for review see Montell 2009). In adult *Drosophila*, putative sugar and putative bitter receptors are expressed in distinct sets of sensory neurons (Wang et al. 2004; Marella et al. 2006; Weiss et al. 2011), and a single sensory neuron typically expresses several *Gr* genes (Thorne et al. 2004; Weiss et al. 2011; this holds true for larvae, too: Kwon et al. 2011). Additionally, it has been reported that different types of bitter-activated neurons, expressing different combinations of GRs, correspondingly respond to different subsets of bitter substances (Meunier et al. 2003; Weiss et al. 2011). Based on *Gr-Gal4* transgene analyses, 39 *Gr* genes (from 68) were shown to be expressed in the taste organs of the larval

head (Colomb et al. 2007; Kwon et al. 2011). From the known bitter-sensitive GRs (*Gr33a*, *Gr66a*, *Gr93a*) expressed in adults (Scott et al. 2001; Thorne et al. 2004; Jiao et al. 2007; Lee et al 2009), *Gr33a* and *Gr66a* are expressed also in the larva, co-expressed with up to 15 other *Gr*s within one cell (Kwon et al. 2011). However, none of the known adult-expressed sugar-sensitive *Gr*s (*Gr5a*, *Gr64a*, *Gr64f*) (Thorne et al 2004; Dahanukar et al. 2007; Jiao et al. 2007, 2008) is found in the larva, such that the molecular basis of sugar detection in larvae, quite distressingly, remains unknown. Beside the *Gr* gene family, receptors of the *TrpA* family (Kim et al. 2011) and/or the *Ir* family (Benton et al. 2009) may participate in gustation in the larva.

Thus, the precise function of most GRs in larvae and the behavioural responses they induce are still unknown. It was shown, however, that they are able to sense different substances tasting sweet (Schipanski et al. 2008; Rohwedder et al. 2012), salty (Niewalda et al. 2008) or bitter (Hendel et al. 2005; El-Keredy et al. 2012) to humans and, like in other animals, sweet and bitter substances are shown to influence choice, feeding and learning behaviour.

Gustatory choice behaviour is thought to be rather rigid and reflexive in nature (Schleyer et al. 2011). However, our knowledge is based on experiments using pure substances. Mixtures of compounds, probably providing a much more natural situation, have been not tested so far; thus the full scope of gustatory perception and processing still remains unresolved. In this study, we test whether larval attraction toward sugar indeed is an inflexible reflex-like behaviour or whether it is sensitive to modifications by gustatory context. Specifically, we ask whether the presence or absence of bitter tastants influences animals' attraction toward sweet tastants.

Material and Methods

Larvae

Third-instar feeding-stage larvae from the *Drosophila melanogaster* wild-type strain Canton-S were used throughout. Every day, a few hundred of adult flies were transferred to fresh standard food vials and allowed to lay eggs for 24 hr at 25°C, 60-70% relative humidity, under a 14/10 hr light/dark cycle. Then, adult flies were removed and vials were kept at the mentioned culture conditions for four additional days until larvae were collected for experiments. A spoonful of medium from a food vial

was taken and larvae were briefly rinsed twice in tap water to remove food residues. Experiments were then started, using cohorts of 15 larvae each.

Dishes

We used Petri dishes of 55 mm inner diameter (Sarstedt, Nümbrecht, Germany), covered one half with a plastic stamp (Fig. 1A) and filled the other side either with freshly boiled agarose solution that, for example, in addition contained a sweet tastant. Once solidified, we removed the stamp and filled the second half with another substrate, for example one with agarose but without an added sweet tastant. After the second half had solidified, we covered the dishes with their lids and left them at room temperature until the experiment started 1-4 hours later; using the Petri dishes so relatively soon after their preparation ensures that diffusion is minimal. Indeed, for some of the bitter tastants we observed that using the Petri dishes the following day degrades avoidance scores (data not shown); no such effects were seen for the sweet tastants.

As substrates we used either pure 1 % aequous agarose solution (Pure) (electrophoresis grade) or agarose in addition containing different sweet and/or bitter tastants at the concentrations mentioned along the Results section. We use fructose (F) (CAS: 57-48-7, purity > 99.5 %), glucose (G) (CAS: 14431-43-7, purity > 99.5 %), sucrose (Su) (CAS: 57-50-1, purity > 99.5 %) or sorbitol (So) (CAS: 50-70-4, purity > 98 %) (all from Roth, Karlsruhe, Germany) as sweet tastants, and quinine hemisulfate (Q) (CAS: 6119-70-6, purity 94 %, Sigma-Aldrich, Seelze, Germany) as bitter tastant.

Choice

For the choice experiments, one half of the Petri dishes contained Pure agarose, while the other half contained either a sweetened or a bitter substrate. In half of the cases, the tastant side was to the left and in the other half of the cases the tastant side was to the right, to average-out spurious effects of the experimental surround. We placed 15 larvae in the middle of these Petri dishes and closed the lid. At the time point(s) mentioned along the Results section, we scored the number of larvae located at either the Pure side, the tastant side, a 1 cm-wide middle stripe (Fig. 1B), or the lid. To calculate a gustatory preference index (PREF), we subtracted the number of larvae on the Pure side ($\#_{Pure}$) from the number

of larvae on the tastant side ($\#_{Tastant}$) and divided this difference by the total number of larvae on the dish (but without the number of larvae on the lid):

(1a) $PREF = (\#_{Tastant} - \#_{Pure}) / \#_{Total}$

Thus, PREF values were constrained between 1 and -1, positive values indicating a preference for and negative values indicating aversion of the tastant.

Fig. 1C shows the time dependency of innate (i.e. experimentally naïve) larvae toward fructose. Larvae were confronted with a choice between PURE agarose and agarose containing 2 M fructose (Fig. 1C). In accord with previous studies (Schipanski et al. 2008; Schleyer et al. 2011; Rohwedder et al. 2012) we found strong attraction toward fructose: Preference scores were positive for all tested time points and increased with time up to 0.8 after 8 minutes. For all following experiments, we therefore restricted data analyses to the 8-minute time point (time-resolved data are presented as Supplemental Material).

Interaction

Interaction experiments were performed in principle as the choice experiments. However, the interaction test always involved two experimental groups:

For the first group, Petri dishes contained a sweet tastant on one side and Pure agarose on the other side (Sweet *versus* Pure), and the preference for the sweet tastant was determined as:

(1b) $PREF = (\#_{Sweet} - \#_{Pure}) / \#_{Total}$

For the second group, Petri dishes also contained a sweet tastant on one side, but a bitter tastant was added to <u>both</u> sides (Sweet+Bitter *versus* Bitter). We then calculated the preference for the sweet tastant-side as:

(1c) $PREF = (\#_{Sweet+Bitter} - \#_{Bitter}) / \#_{Total}$

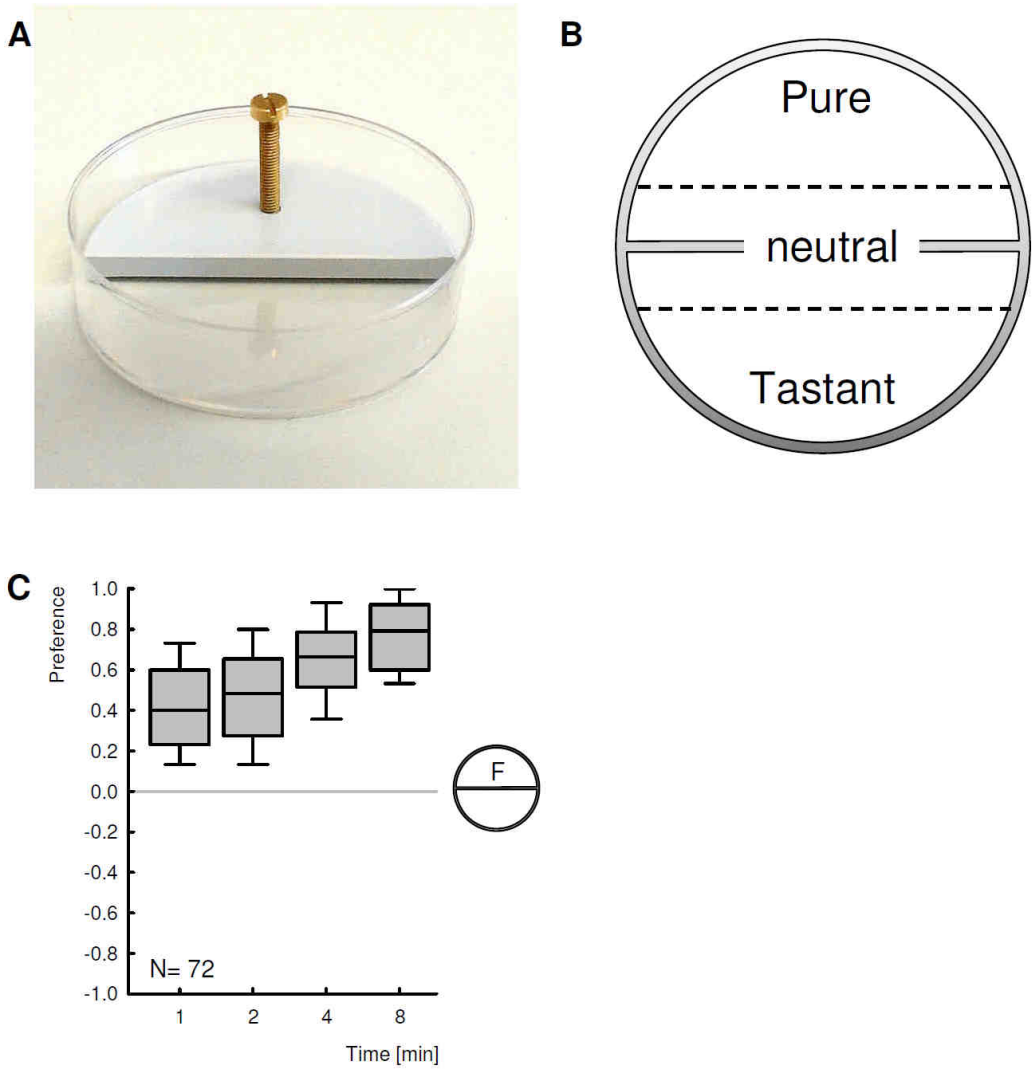

Figure 1: *Gustatory choice*
(A) Plastic stamp to prepare Petri dishes with different substrates on either half.
(B) Schematic of the experimental setup and scoring. Cohorts of 15 larvae were allowed to distribute between the halves of a Petri dish filled for example with agarose and an added tastant on one side (Tastant), versus agarose-only on the other side (Pure); larvae remaining within a middle zone were scored separately. Gustatory preference scores were then determined as the number of larvae on the tastant side minus the number on the other side, divided by the total number of larvae scored.
(C) Gustatory preference scores for fructose (F) calculated based on the distribution of larvae 1, 2, 4 and 8 minutes after assay onset. Based on these results, we restrict data display of the following experiments to the 8-min time point in order be able to measure reductions of preference in a graded manner (time-resolved data for the following experiments can be found in the Supplementary Material).
Shaded boxes indicate significant differences from zero ($P< 0.05/4$, OSS). Box plots represent the median as the middle line, 25 / 75 % quantiles as box boundaries and 10 / 90 % quantiles as whiskers, respectively.

Thus, again preference for the sweet-containing side shows by positive PREF scores. We reasoned that if there is no interaction between bitter and sweet processing, both groups should behave the same because in both groups it is the presence of the sweet tastant that makes the difference between the two sides of the Petri dish. An inhibitory interaction, however, would show by the preference for the sweet-containing side being lessened in the background of a bitter tastant.

We note that although this experimental design does allow testing whether there is an inhibitory interaction, it does not allow conclusions about the level of processing at which such interaction takes place (see Discussion).

Statistical analyses

All statistical analyses were performed with Statistica on PC. Preference values were compared across multiple groups with Kruskal-Wallis tests. For subsequent pair-wise comparisons, Mann-Whitney U-tests were used. To test whether values of a given group differ from zero, we used one-sample sign tests. When multiple tests were performed within one experiment, we adjusted significance levels by a Bonferroni correction to keep the experiment-wide error rate at 5 %. This was done by dividing the critical *P*-value of 0.05 by the number of tests. Data are presented as box plots which represent the median as the bold middle line and 25 % / 75 % and 10 % / 90 % quantiles as box boundaries and whiskers, respectively.

Results

Quinine inhibits fructose processing

First, we tested for an interaction between bitter and sweet processing by comparing two groups of larvae (Fig. 2A): One group was confronted with a choice of fructose *versus* pure agarose, while for the second group fructose preference was tested in the background of quinine (fructose+quinine *versus* quinine). Quinine tastes bitter to humans and is reported to be aversive to adult (e.g. Meunier et al. 2003; Weiss et al. 2011) and larval *Drosophila* (Hendel et al. 2005; El-Keredy et al. 2012). If there is no interaction between fructose and quinine processing, that is if the behavioural tendencies regarding fructose and quinine would merely add up, both groups should behave the same. This is because in both groups the difference between the two sides of the Petri dish is the same: The presence *versus* absence of fructose. However, the preference for the fructose-containing side was less in the background of quinine (Fig. 2A), suggesting that quinine inhibits fructose processing. This inhibition of quinine onto fructose processing was partial for a relatively high concentration of fructose (Fig. 2A: 2 M); expectedly, as fructose concentration was reduced, fructose preferences were less strong and

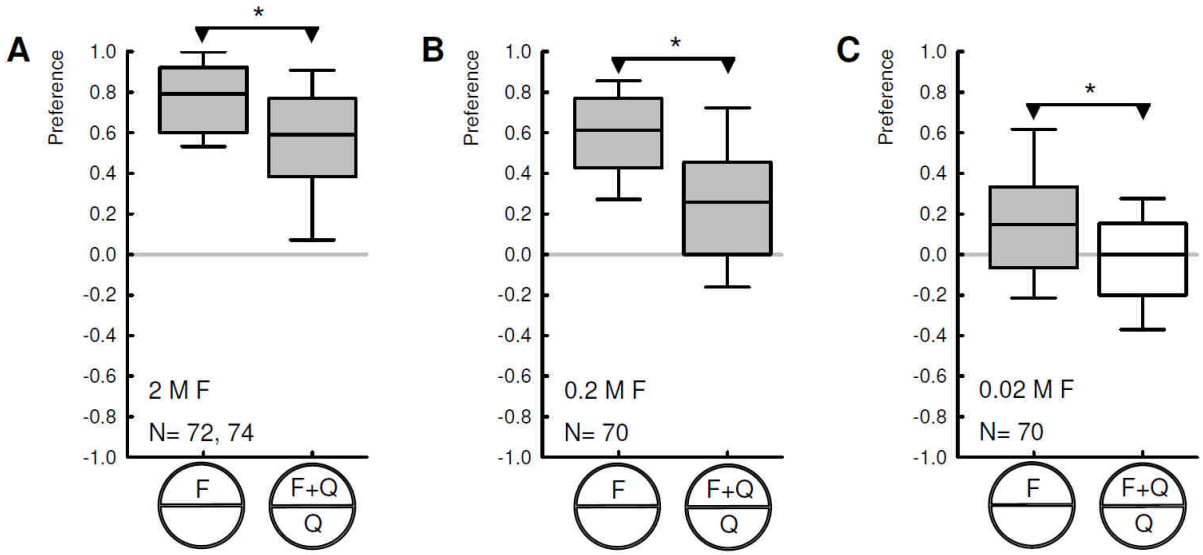

Figure 2: *Quinine inhibits fructose processing*
(A) In both groups, the presence of fructose (F, 2 M) is the sole difference between the halves of the Petri dish. If there were no interaction between quinine (5 mM) and fructose processing, the larvae should thus distribute themselves according to this differential, leading to equal levels of preference for the fructose-containing half in both groups. However, fructose preference is lessened in the background of quinine ($P< 0.05$, U= 1522, MWU) suggesting that quinine inhibits fructose processing.
(B, C)Using lower concentrations of fructose, the same inhibition effect is seen, at respectively lower over-all levels of preference (B: 0.2 M, $P< 0.05$, U=993, MWU; C: 0.02 M, $P< 0.05$, U=1536.5, MWU).
Shaded boxes indicate significant differences from zero ($P< 0.05/ 2$, OSS). Significant differences between groups are shown with an asterisk over the bold line ($P< 0.05$; MWU). Note that the data of B) and C) are also included in Fig. 4A and B, respectively. For further details see legend of Fig. 2.

these less strong preference scores could be inhibited fully (Fig. 2B, C). More importantly, inhibition was getting stronger with increasing quinine concentration (Fig. 3A, B): The differential presence of fructose mattered the less the more quinine was present.

We conclude that quinine and fructose processing interact, such that quinine inhibits fructose processing in a concentration-dependent manner. Obviously, the level of processing at which this inhibition takes place must remain unspecified at this point.

Which other sweet tastants can quinine inhibit?

Next, we asked for the stimulus-specificity of this inhibitory effect of quinine. Specifically, we asked upon which sweet tastants quinine could exert inhibition. To allow 'fair' comparisons of the degree of quinine inhibition onto processing of different sweet tastants, we first determined the dose-response functions for preference toward four sweet tastants (Fig. 4 A-D), namely for the three sugars fructose (F), glucose (G), and sucrose (Su), as well as for one artificial sweetener, sorbitol (So). We then chose concentrations such that they supported preference scores of approximately 0.5 in all four cases

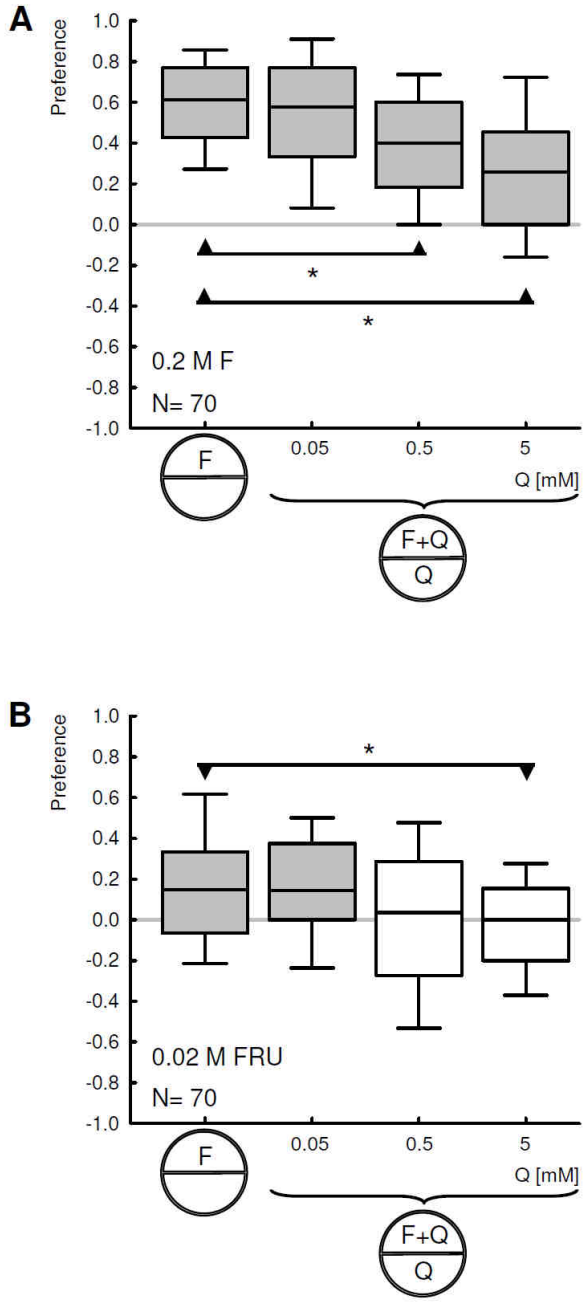

Figure 3: *Quinine inhibits fructose processing: Dependence on quinine concentration.*
(A) Increasing quinine concentration (from left to right: 0.05 mM; 0.5 mM; 5 mM) increases the inhibitory effect of quinine onto fructose (0.2 M) processing. A comparison across groups reveals significant differences across quinine concentrations ($P< 0.05$, H= 48.91, df= 3, KW). Inhibition is significant for 5 mM quinine ($P< 0.05/ 3$, U= 993, MWU), 0.5 mM quinine ($P< 0.05/ 3$, U=1359, MWU), but not for 0.05 mM quinine ($P= 0.46$, U= 2274.5, MWU).
Shaded boxes indicate significant differences from zero ($P< 0.05/ 4$, one-sample sign-tests). Significant differences between groups are shown as asterisk over the bold line ($P< 0.05/ 3$, Mann-Whitney U tests).
(B) As in (A), using 0.02 M fructose. A comparison across groups reveals significant differences across quinine concentrations ($P< 0.05$, H= 21.4, df= 3, KW). Inhibition is significant for 5 mM quinine ($P< 0.05/ 3$, U= 1536.5, MWU), but for 0.5 mM quinine ($P= 0.02$, U= 1887.5, MWU) and 0.05 mM quinine ($P= 0.93$, U= 2429.5, MWU). For further details see legends of Fig.s 2, 3.

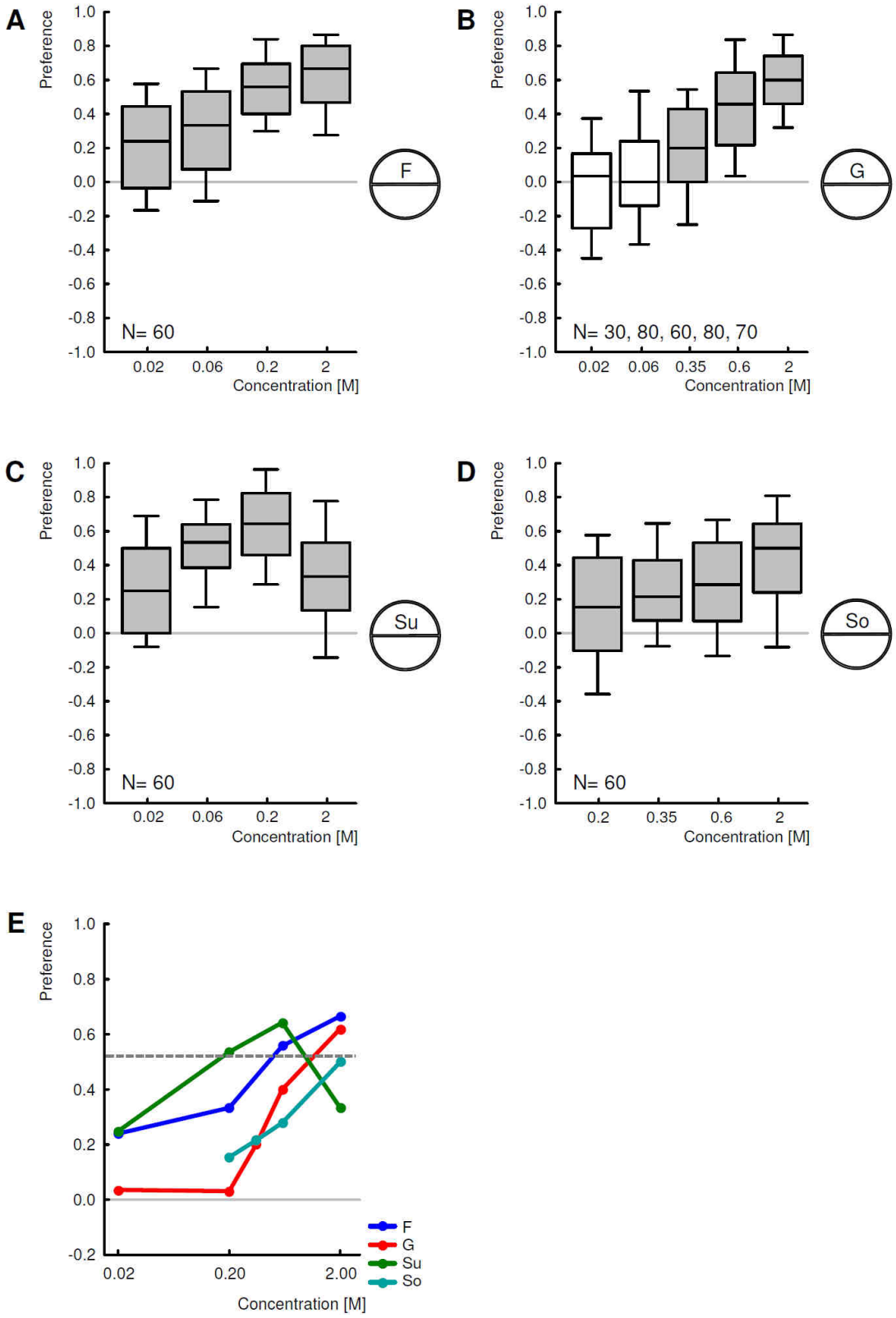

Figure 4: *Dose-response functions* for the preference for (A) fructose (F), (B) glucose (G), (C) sucrose (Su), and (D) sorbitol (So). E) Overview, using the median preference score for the respective tastant and concentration. The dashed line represents a level of comparable preference (Preference= 0.5), such that 'fair' concentrations of these tastants can be used for the Experiment in Fig. 5.
Shaded boxes indicate significant differences from zero (A, C, D: $P< 0.05/4$; B: $P< 0.05/5$; OSS). For further details see legends of Fig. 2.

(dashed line in Fig. 4E). This allowed us to ask how strongly quinine could inhibit these equally-preferred sweet tastants. It turned out that for glucose inhibition was almost complete, for fructose and sorbitol inhibition was partial, while for sucrose no inhibition at all was observed (Fig. 5).

We conclude that quinine inhibits processing of sweet tastants in a stimulus-specific way: Glucose is particularly susceptible to quinine-inhibition, fructose and sorbitol less so, while sucrose is strikingly unaffected.

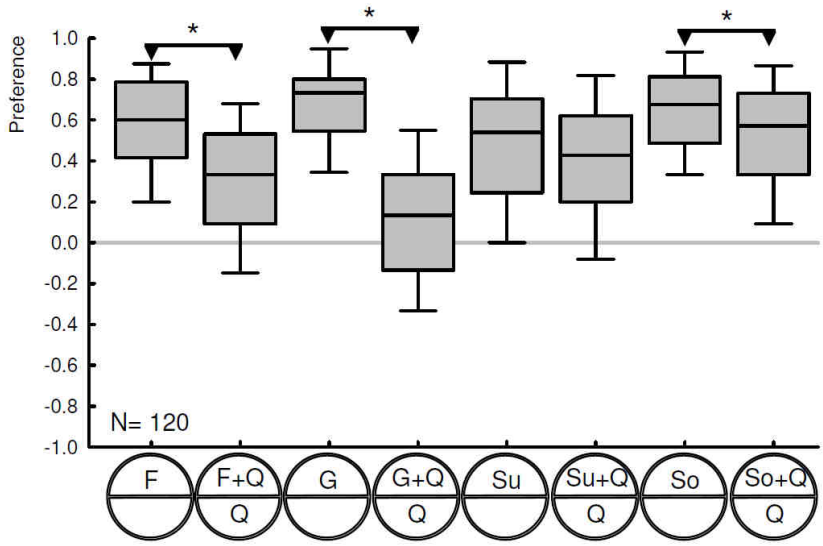

Figure 5: *Inhibition of various sweet tastants by quinine.*
Gustatory preferences in choice between pure agarose and agarose containing 0.2 M fructose (F), 1.24 M glucose (G), 0.02 M sucrose (Su) or 2 M sorbitol (So), and between 5 mM quinine (Q) against quinine plus the respective sweet tastant (F+Q, G+Q, Su+Q, So+Q). A comparison across groups reveals significant differences between groups ($P< 0.05$, H= 245.2, df= 7, Kruskal-Wallis H-test). The presence of quinine results in statistically different levels of gustatory preference in case of F, G and So (P< 0.05/ 4, from left to right: U= 3662, 1253, 5259, Mann-Whitney U-tests), but not in case of Su (P> 0.05/ 4, U= 6076). Shaded boxes indicate significant differences from zero ($P< 0.05/ 8$, OSS). Significant differences between groups are shown as asterisk over the bold line ($P< 0.05/ 4$, Mann-Whitney U-tests). Note that no time-resolved data exist for this experiment, as larvae were scored only after 8 min. For further details see legends of Fig. 2.

Discussion

Behaviour toward sweet tastants

We find strong attraction toward sweet tastants which correlates, despite some difference in methods, with results of previous studies (Schipanski et al. 2008; Rohwedder et al. 2012). Specifically, we find that maximal attraction toward fructose, glucose and sucrose is similarly high and toward sorbitol is relatively lower. This corresponds to reported nutritional values of these sweet tastants (Rohwedder et al. 2012). However, larvae are highly sensitive to fructose and sucrose, with a detection threshold of below 0.02 M (Fig. 4E) (Schleyer et al. 2011 reported a threshold of approx. 0.01 M in case of

fructose). In contrast, responses to glucose and sorbitol are seen only at an approx. one order of magnitude higher concentration (Fig. 4E). Thus, the detection thresholds to sweet tastants seems to be not correlated with the nutritional value but with another feature of the tastants like, for example, their sweetness. Notably, fructose and sucrose taste relatively sweeter than glucose to humans and were shown to be detected at lower concentrations (Pangborn 1963). However, it is not clear how to determine the "sweetness" of tastants as perceived by the larvae. In adult flies, labellar gustatory sensilla show lower response rates to fructose than to glucose or sucrose (Dahanukar et al. 2007), but their detection thresholds were not analysed.

We note that attraction to sucrose reaches its maximum at a lower concentration than fructose or glucose, and is then decreasing as concentration is further increased. Previous studies report such optimum-like functions for all sweet tastants used here (Schipanski et al. 2008; Rohwedder et al. 2012), and it is possible that we could find similar effects for the other sweet tastants if we would extend the range of tested concentrations. However, such decreased preferences at very high sugar concentrations may be based on non-gustatory processing related to the consistency of the sucrose-agarose gel (small droplets arising all over the surface were observed when the agarose is cooling down). Such potential effects of changing consistency may show up at rather low concentrations in the case of sucrose because the molecular weight of sucrose is approx. twice as high as compared with the other sugars used, and therefore the amount of sugar in the agarose is doubled (for 2 M sucrose solution, as much as 68 g sucrose is dissolved in a total volume of 100 ml).

Inhibitory effect of quinine

In adult *Drosophila*, it is known that flies' attraction toward sugars can be reduced by the presence of bitter tastants (e.g. Meunier et al. 2003). Indeed, as bitter sensory cells were unknown for a long time, it was assumed that the behavioural response of fruit flies to bitter tastants might be mostly or exclusively mediated by inhibition sugar processing (Morita and Yamashita 1959; Tanimura and Kikuchi 1972; Siddiqi and Rodrigues 1980).

In 2003, gustatory sensilla that respond to bitter tastants were found and described for the first time by Meunier and colleagues. They found that quinine and other bitter substances have a threefold

effect: (i) They activate so called ‘L2’-cells (which are also activated by high concentrations of salt), and they inhibit both (ii) water-sensitive ‘W’-cells and (iii) sweet-sensitive ‘S’-cells (Meunier et al. 2003). Concerning the following discussion, two aspects regarding sugar inhibition in adults are important to note:

First, gustatory sensilla typically respond to many bitter tastants (but not to e.g. sweet tastants) with a delay of up to several hundred milliseconds (Meunier et al. 2003; Weiss et al. 2011); a similar delay is found for the inhibitory effect of quinine on sucrose processing, too (Meunier et al. 2003). Notably, when recording from a sensillum housing both a L2- and a S-cell and exposing it to a mixture of sucrose and quinine, the S-cell stops firing exactly when the L2-cell starts to do so (Meunier et al. 2003 *loc. cit.* Fig. 5D). This suggests, at least for the tarsal sensilla, a common mechanism for an (delayed) activation of the bitter-sensitive cell and the inhibition of the sweet-sensitive cell (Fig. 6A)

Second, in a gustatory sensillum housing only a S-cell but no L2-cell (the so-called f2b and f3b sensilla, respectively), thus featuring no bitter-activated neuron, the sugar response can be inhibited by quinine (Meunier et al. 2003 *loc. cit.* Fig. 5C). Thus, at least in adult tarsal sensilla, no bitter-activated neuron is required for inhibition of sugar signals (Fig. 6B-B’’).

Thus, at present two basic scenarios of how quinine inhibits sugar processing remain conceivable, both operating relatively far out in the sensory periphery: Either inhibition is indirect, in the sense that it is based on the action of a bitter-sensitive neuron onto the sugar neuron (Fig. 6A), or it is direct, i.e. based on the action of the bitter tastant itself on the sugar neuron (Fig. 6B-B’’). Possibly, both processes have to be reckoned with, albeit at different sensilla.

In the current study, we find that quinine is able to reduce the attraction of three of the four tested sweet tastants – however, to different extents: Sucrose is not significantly inhibited by quinine, and glucose is most prone to be inhibited (Fig. 5). As all sweet tastants were used at concentrations that induce similar levels of attraction, these differences in susceptibility to inhibition seem to reflect genuinely stimulus-specific differences. That is, the mechanism of inhibition acts differentially depending on the identity of the sweet tastant in question. Interestingly, it has been shown that in adults glucose is sensed by the GR5a receptor molecule, whereas fructose and sucrose are sensed by GR64a (Dahanukar et al. 2007). However, both these sugar receptor molecules in adults are usually

expressed in the same cells (Weiss et al. 2011). This implies that although the identity of the sweet tastant could be discerned using two differentially tuned receptor molecules, such discriminative power is abandoned at the level of the sensory neuron. If a similar organisation would be found in the larva, the found differences between sweet tastants in their sensitivity to inhibition may hint at a relatively peripheral site of inhibition, i.e. at a point of processing before the signals carried by the respectively expressed GRs converge (Fig. 6B, B').

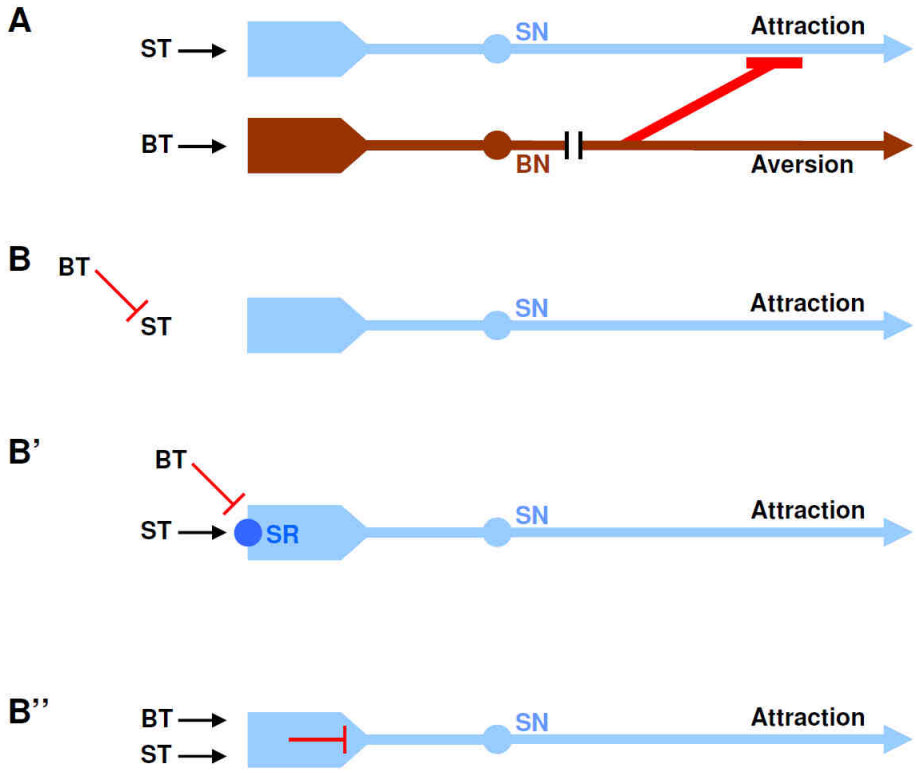

Figure 6: *Models of inhibition.*
A-D show different suggestions about potential locations of the inhibitory effect of quinine in the larval chemosensory system.
(A) The sweet sensory neuron may be inhibited by the bitter sensing pathway. Specifically, a BT activates a bitter sensory cell (BN) and the bitter signal is passed through the gustatory pathway finally steering bitter-related behaviour. A ST activates a sweet-sensory cell (SN) but its activity then is inhibited by the bitter pathway.
(B) Bitter (BT) and sweet tastants (ST) may interact with each other at molecular level in the substrate, such that e.g. the presence of a BT influences the binding of a ST to its receptor molecule.
(B') BTs may compromise the function of the sugar receptor molecule (SR). Indeed, it has been shown in adults that CO_2-induced avoidance behaviour can be inhibited by a particular class of odours directly acting on the CO_2-receptor (coded by *Gr21a* and *Gr63a*: Turner and Ray 2009).
(B'') A BT may act within sweet-sensitive gustatory receptor neurons (SN), i.e. at an intracellular domain of the SR, or downstream of the SR. Quinine is reportedly able to enter artificial liposomes and rat taste cells (Perry et al. 2000) and to directly activate G-proteins in liposomes (Naim et al. 1994).

Obviously, the identification of the receptor molecules and cells of the larva that are underlying sweet- and bitter-processing is required before experiments to scrutinise this suggestion can be devised. This study nevertheless provides the framework for future research on the surprisingly flexible choice behaviour of larval *Drosophila* in order to further analyse the cellular and molecular mechanism of inhibition.

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Supplement

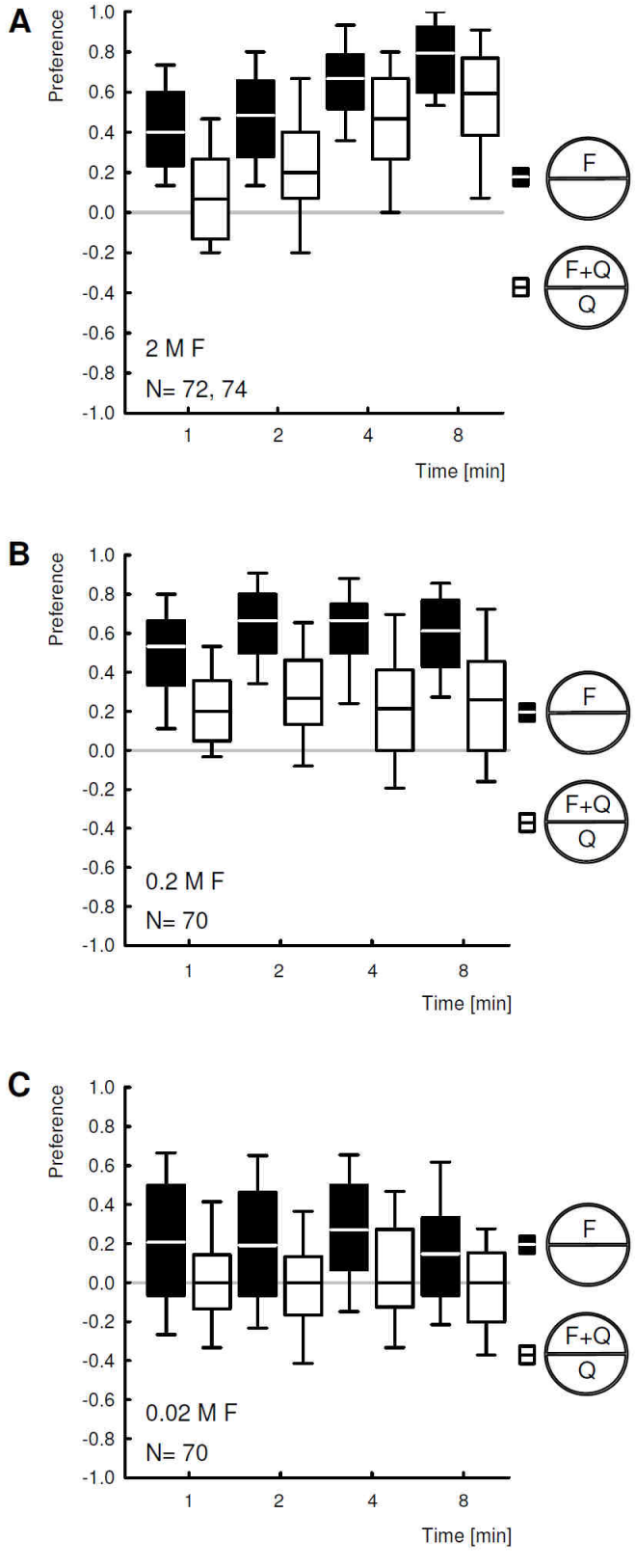

Figure S1: Time course of preference scores based on the distribution of larvae 1, 2, 4 and 8 minutes after assay onset. This display relates to Figure 2, which shows the 8-minute data only.

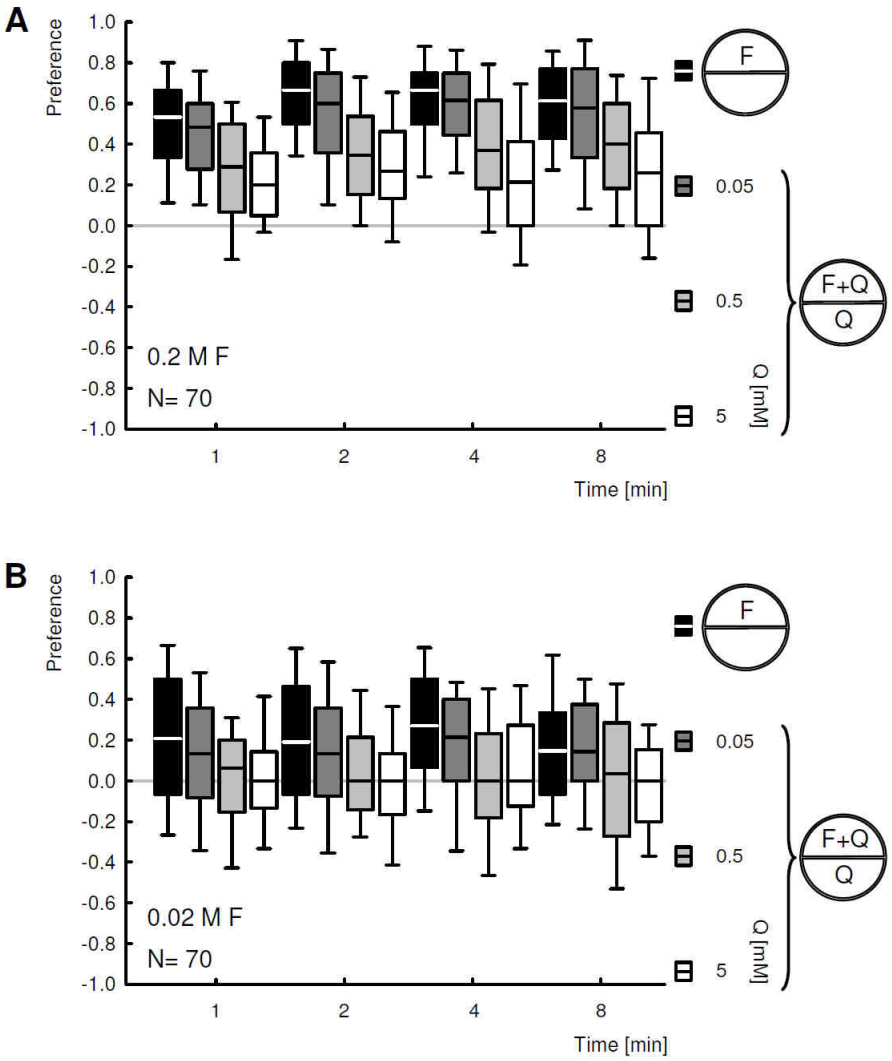

Figure S2: Time course of preference scores based on the distribution of larvae 1, 2, 4 and 8 minutes after assay onset. This display relates to Figure 3, which shows the 8-minute data only.

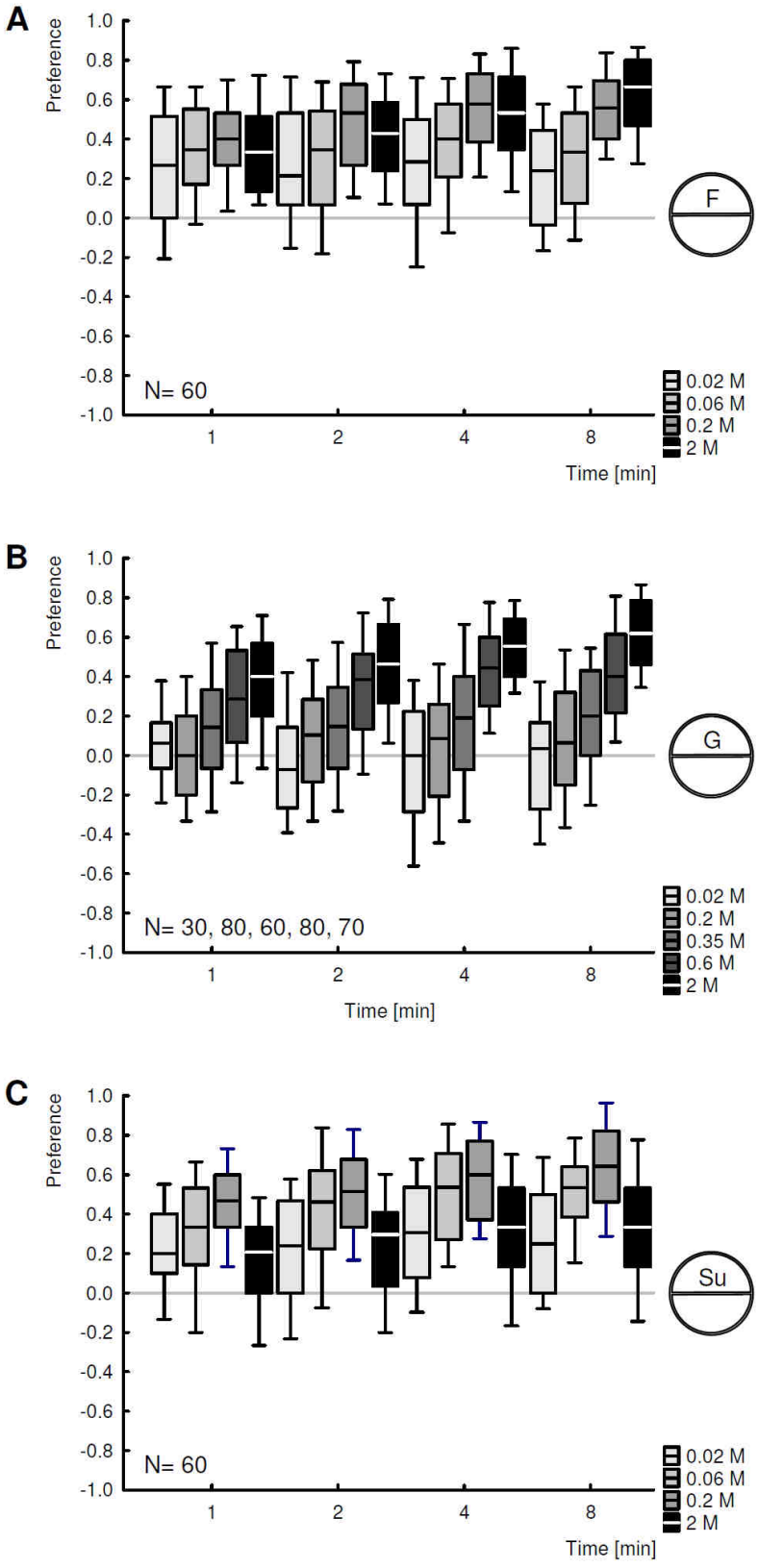

Figure S3: For figure legend, see next page

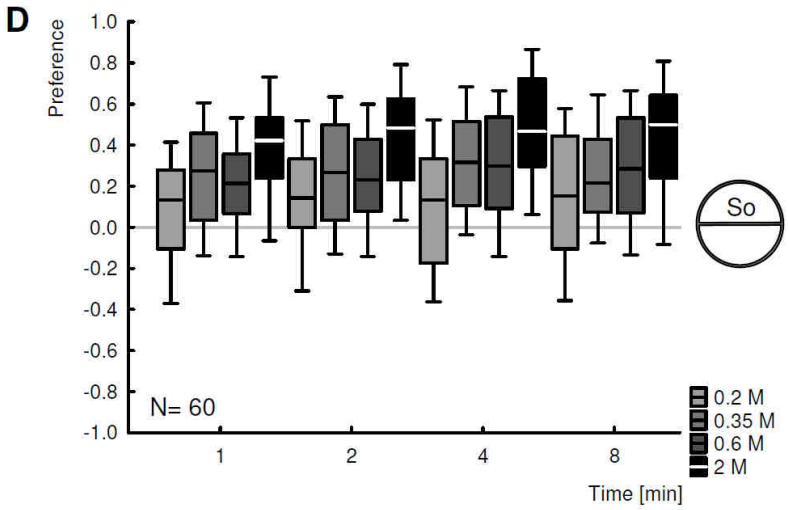

Figure S3 (continued)**:** Time course of preference scores based on the distribution of larvae 1, 2, 4 and 8 minutes after assay onset. This display relates to Figure 4, which shows the 8-minute data only.

Outlook

Is behaviour response or action? In this Thesis, I studied this question with regard to learned and innate behaviour and found learned behaviour to be active and driven by outcome expectations (chapter I). Innate behaviour, in turn, seems to be more responsive and less open to environmental signals but still allows for some flexibility (chapter II). On the following pages, I discuss perspectives and strategies for further research on the topics treated in this Thesis.

The 'content' of the larva's expectations

Having found evidence for expectation-driven behaviour in larval *Drosophila* (section I.1), an interesting question is what the 'content' of these expectations is. When it smells the previously fructose-rewarded odour, does a larva expect to find reward? Does it expect sugar? Or fructose? Does it expect reward/sugar/fructose at a particular intensity? This problem is entangled with the question of how 'rich' the memory trace is, that means how much information it contains. Previous studies showed that an odour-taste-memory trace features information about the identity (Chen et al. 2011) and intensity (Mishra et al. submitted; regarding adults: Yarali et al. 2009) of the trained odour. In my work I found that, at least with respect to fructose, the memory trace also features information about the intensity of the gustatory reinforcer. The open question is whether it contains information about the *reinforcer identity*, too. With the experimental procedures and conceptual framework developed in this Thesis, this can now be tested: For example, one could train the larvae with reward A and perform the test in presence of another reward B. A second group of larvae is handled *vice versa*, that is, they are trained with reward B and tested in presence of reward A. If animals behaviourally express their memory in presence of the not-trained reward, this hints to reward-specific memory traces. Such a finding would result in far reaching claims for the neuronal architecture underlying memory trace formation and retrieval.

A practical challenge for such kinds of experiments is to find several appetitive reinforcers that are similarly potent and can be discriminated by the larva on the sensory level. To this regards, aversive learning provides a larger variety of known, rather distinct reinforcers such as salty taste

(Gerber and Hendel 2006; Niewalda et al. 2008; section I.1), mechanical disturbance (Eschbach et al. 2011), electrical shock (Khurana et al. 2009; Pauls et al. 2010a) or heat (Khurana et al. 2012). Thus, one could perform experiments analogous to those described above with aversive learning, testing whether a punishment A can enable the expression of a memory trace formed with punishment B.

Neuronal architecture of decision-making

An obvious issue for future research is to find the cells involved in the process of comparison and decision-making. As location of the memory trace in adult and larval *Drosophila* the mushroom-body Kenyon cells are identified (regarding larva: Honjo and Furukubo-Tokunaga 2005, 2009; Selcho et al. 2009; Pauls et al. 2010b; Michels et al. 2011; regarding adults: Riemensperger et al. 2005; Claridge-Chang et al. 2009; reviews by Heisenberg et al. 2003; Gerber et al. 2004, 2009), being coincidently activated by the odour signal and the reinforcer signal *via* octopaminergic and/or dopaminergic neurons, respectively (Schwaerzel et al. 2003; Schroll et al. 2006, Honjo and Furukubo-Tokunaga 2009; Selcho et al. 2009; for a rather detailed overview, see section I.3 of this thesis). As the comparison between 'observed' and 'expected' value requires the memory trace to be activated, the location of the comparison has to be downstream of the mushroom body; and obviously is has to be upstream of the neuromuscular synapse finally driving larval movement. The functional connection between mushroom body and motor commands is presently a big black box, and to unravel these connections is one of the big issues of future research in the field. Finding the neurons responsible for the decision whether to behaviourally express a memory or not might be one important step to close the gap between memory trace and learned behaviour.

Conceivably, one can try to close this gap from two sides: Coming from the mushroom body as site of the memory trace, one can search 'downstream' for mushroom body extrinsic neurons that are specifically involved in the signalling of the activated memory trace toward motor control and/or in the signalling of the current gustatory situation (e.g. sugar/no sugar). This could be done by genetically disabling single, or subsets of, mushroom body extrinsic neurons during a learning experiment. Such experiments are currently being performed as part of the so called 'larval olympiade' at the Janelia Farm Research Campus (T. Saumweber and B. Gerber, LIN Magdeburg, pers. comm.), such that new

insights into this topic will likely be available soon. Coming from the behaviour as ultimate consequence of the decision process, one can perform temporally and locally fine grained observations to find those motor patterns that are changed in animals expressing learned behaviour compared to animals that show only innate behaviour. From such changed motor patterns one can work 'upstream' toward the central pattern generators at the level of premotor neurons and their connection and/or their susceptibility to centrifugal neuromodulation upon the activation of the memory trace.

Localisation of the inhibitory effect of bitter tastants

Regarding the topic of bitter-sweet interactions (section II.1), it would be worthwhile to further investigate the inhibitory effect of quinine on larval sugar attractiveness and to search for additional substances (e.g. other bitter substances) that induce similar effects: Insect larvae regularly cause huge damages to crops worldwide. Revealing a mechanism to reduce the attractiveness of food or animals' appetite for it could be of enormous benefit for agriculture.

The next step to study the inhibitory effect would be to localise it within the gustatory processing pathway. In section II.2, we provide several possible scenarios: Bitter substances may either act on the sugar molecules themselves, the sugar sensory cell (either directly at the receptor or *via* intracellular pathways), or the bitter sensory cells such that bitter processing inhibits sugar processing. The most promising approach to determine the appropriate scenario certainly is to take advantage of the repertoire of tools available to genetically manipulate larval neurons. The gustatory receptors GR66a and GR33a are thought to be expressed in all bitter-activated gustatory neurons (Thorne et al. 2004; Marella et al. 2006; Weiss et al. 2011; Kwon et al. 2011). Driving *Gr66a*-expressing cells induces aversive behaviour in larvae (Colomb et al. 2007). Preventing those cells from firing during an inhibition experiment will reveal whether bitter-activated neurons are *necessary* for this inhibitory effect. Driving *Gr66a*-expressing cells while measuring animals' sugar preference, in turn, will reveal whether those neurons are *sufficient* to induce inhibition. In case bitter-activated neurons are found to be *not* involved in inhibition, we would suggest that quinine most likely acts directly on the sugar-activated neuron (despite of the possibility that a third type of neurons, being neither *Gr66a*-expressing nor sugar-activated, might be involved). The challenge for future research

would then be to disentangle whether the inhibitory effect takes place *via* directly inhibiting the sugar receptor or *via* other pathways.

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Summary

Is behaviour response or action? In this Thesis I study this question regarding a rather simple organism, the larva of the fruit fly *Drosophila melanogaster*. Despite its numerically simple brain and limited behavioural repertoire, it is nevertheless capable to accomplish surprisingly complex tasks.

After association of an odour and a rewarding or punishing reinforcement signal, the learnt odour is able to retrieve the formed memory trace. However, the activated memory trace is not automatically turned into learned behaviour: Appetitive memory traces are behaviourally expressed only in *absence* of the rewarding tastant whereas aversive memory traces are behaviourally expressed in the *presence* of the punishing tastant. The 'decision' whether to behaviourally express a memory trace or not relies on a quantitive comparison between memory trace and current situation: only if the memory trace (after odour-sugar training) predicts a stronger sugar reward than currently present, animals show appetitive conditioned behaviour. Learned appetitive behaviour is best seen as active *search for food* – being pointless in the presence of (enough) food. Learned aversive behaviour, in turn, can be seen as *escape from a punishment* – being pointless in absence of punishment. Importantly, appetitive and aversive memory traces can be formed and retrieved independent from each other but also can, under appriate circumstances, summate to jointly organise conditioned behaviour.

In contrast to learned behaviour, innate olfactory behaviour is not influenced by gustatory processing and *vice versa*. Thus, innate olfactory and gustatory behaviour is rather rigid and reflexive in nature, being executed almost regardless of other environmental cues.

I suggest a behavioural circuit-model of chemosensory behaviour and the 'decision' process whether to behaviourally express a memory trace or not. This model reflects known components of the larval chemobehavioural circuit and provides clear hypotheses about the kinds of architecture to look for in the currently unknown parts of this circuit.

The second chapter deals with gustatory perception and processing (especially of bitter substances). Quinine, the bitter tastant in tonic water and bitter lemon, is aversive for larvae, suppresses feeding

behaviour and can act as aversive reinforcer in learning experiments. However, all three examined behaviours differ in their dose-effect dynamics, suggesting different molecular and cellular processing streams at some level.

Innate choice behaviour, thought to be relatively reflexive and hard-wired, nevertheless can be influenced by the gustatory context. That is, attraction toward sweet tastants is decreased in presence of bitter tastants. The extent of this inhibitory effect depends on the concentration of both sweet and bitter tastant. Importantly, sweet tastants differ in their sensitivity to bitter interference, indicating a stimulus-specific mechanism.

The molecular and cellular processes underlying the inhibitory effect of bitter tastants are unknown, but the behavioural results presented here provide a framework to further investigate interactions of gustatory processing streams.

Zusammenfassung

Ist Verhalten Aktion oder Reaktion? In dieser Arbeit widme ich mich dieser Frage anhand eines recht einfachen Organismus, der Larve der Taufliege *Drosophila melanogaster*. Trotz ihres nur aus wenigen Tausend Nervenzellen bestehenden Gehirns und begrenzten Verhaltensrepertoires ist sie dennoch zu überraschend komplexem Verhalten fähig.

Nach der Assoziation eines Duftes mit einem belohnenden oder bestrafenden Geschmacksstoff ist der gelernte Duft in der Lage, die gebildete Gedächtnisspur abzurufen. Diese aktivierte Gedächtnisspur wird jedoch nicht automatisch in Verhalten übersetzt: Appetitive Gedächtnisspuren führen nur *in Abwesenheit des belohnenden Geschmacks* zu erlerntem Verhalten, während aversive Gedächtnisspuren nur *in Anwesenheit des bestrafenden Geschmacks* in erlerntem Verhalten münden. Die „Entscheidung", eine Gedächtnisspur in Verhalten zu übersetzen oder nicht, beruht auf einem quantitativen Vergleich zwischen der Gedächtnisspur und der aktuellen Situation: Nur wenn die Gedächtnisspur (nach einem Duft-Zucker-Training) eine größere Zuckerbelohnung vorhersagt als gegewärtig vorhanden, zeigen die Tiere appetitives erlerntes Verhalten. Solches Verhalten kann man am besten als aktive *Suche nach Nahrung* interpretieren, die in Gegenwart von (ausreichend) Nahrung sinnlos ist. Aversives erlerntes Verhalten andererseits kann als *Flucht vor einer Bestrafung* verstanden werden – und in Abwesenheit einer Bestrafung gibt es nichts, wovor man fliehen könnte. Appetitive und aversive Gedächtnisspuren können unabhängig voneinander gebildet und abgerufen werden, können unter den richtigen Umständen aber auch gemeinsam erlerntes Verhalten organisieren.

Im Gegensatz zu erlerntem Verhalten wird angeborenes olfaktorisches Verhalten nicht durch das Geschmackssystem beinflusst – und umgekehrt. Angeborenes Verhalten erscheint also relativ starr und reflexhaft und läuft größtenteils unbeeinflusst von anderen Umwelteinflüssen ab.

Schließlich entwerfe ich ein auf Verhalten basierendes Schaltkreismodell des chemosensorischen Systems der Larve und der „Entscheidung", eine Gedächtnisspur in Verhalten umzusetzen oder nicht. Dieses Modell stellt bekannte Komponenten des Systems dar und macht klare Vorhersagen über die Architektur, die bisher noch unbekannte Komponenten haben sollten.

Das zweite Kapitel der Arbeit behandelt die Wahrnehmung und Verarbeitung von (hauptsächlich bitteren) Geschmacksstoffen. Chinin, der bittere Geschmack in Getränken wie Bitter Lemon, wirkt abstoßend auf Larven, unterdrückt ihr Fressverhalten und kann in Lernexperimenten als Bestrafung wirken. Allerdings unterscheiden sich alle drei untersuchten Verhalten in der Dynamik ihrer Dosis-Wirkungskurven, was unterschiedliche molekulare und zelluläre Wirkungsweisen nahe legt.

Angeborenes Wahlverhalten, das als reflexhaft und starr gilt, kann dennoch durch den gustatorischen Kontext beeinflusst werden. Das bedeutet, die Anwesenheit eines Bitterstoffes ist in der Lage, die angeborene Präferenz von Larven für süße Geschmackstoffen zu unterdrücken. Dieser inhibitorische Effekt hängt sowohl von der Konzentration der süßen als auch der bitteren Substanz ab. Was noch wichtiger ist: Die verschiedenen Zucker sind unterschiedlich anfällig für die Störung durch Bitterstoffe, was auf einen Stimulus-spezifischen Mechanismus hindeutet.

Die genauen molekularen und zellulären Prozesse, die diesem inhibitorischen Effekt von Bitterstofen zugrunde liegen, sind noch nicht bekannt, die hier präsentierten Ergebnisse bieten aber einen geeigneten Rahmen für weitergehende Untersuchungen der Interaktionen zwischen verschiedenen Teilen des Geschmacksapparates.

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Curriculum vitae

Michael Schleyer

Personal Information

Date of birth:	31/03/1984
Nationality:	German
Gender:	Male

Education and Employment

1994-2003	High school Armin-Knab-Gymnasium Kitzingen, Germany
2003-2009	Student of Biology University of Würzburg, Germany Main subjects: Genetics, Behavioural science, Ecology
2009	Diploma, Biology (s.c.l.) Diploma Thesis: "Formation and expression of olfactory memory in fruit fly larvae: a behaviour-based model" Supervisor: Bertram Gerber
2009-2012	PhD student in *Drosophila* Neurobiology and Genetics University of Würzburg / University of Leipzig, Germany Supervisor: Bertram Gerber
2010-2012	*Ad personam* PhD fellowship of the Studienstiftung des deutschen Volkes

Practical skills and experiences

Practical skills:	Designing and conducting behavioural experiments Immunohistology (Western Blotting, Whole-mount staining) Basic computer knowledge (Statistical and office programs) Scientific writing
Education:	Practical courses and seminars for undergraduate students Supervision of a foreign PhD student Giving talks and presentations
Language skills:	German (native) and English (advanced)

List of Publications

Schleyer M, Saumweber T, Nahrendorf W, Fischer B, von Alpen D, Pauls D, Thum A, Gerber B. 2011. A behavior-based circuit model of how outcome expectations organize learned behavior in larval *Drosophila*. *Learn Mem* **18**: 639-653.

El-Keredy A[=], Schleyer M[=], König C, Ekim A, Gerber B 2012. Behavioural analyses of quinine processing in choice, feeding and learning of larval *Drosophila*. *PloS ONE* **7** (7) e40525.

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lässt mein Gedächtnis mich im Stich?
Manches ist schon so lange her,
kenn ich nicht alle Namen mehr,
so kenn ich die Gesichter doch
und ich erinnere mich noch
und widme euch nicht weniger Raum.
Geschrieben haben wir uns kaum,
denn eigentlich ging keiner fort,
in einer Geste, einem Wort,
in irgendeiner Redensart
lebt ihr in meiner Gegenwart!“

Reinhard Mey (Wer sonst?), 1970

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