Factors Influencing Reinforcer Properties in Olfactory Associative Learning and Memory in *Drosophila melanogaster* Larvae and Adults

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If nothing we do matters, then all that matters is what we do.

Angel – Season 2, Episode 16, “Epiphany”

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Abstract

Drosophila larvae show attraction towards ethanol, which they mostly encounter in the wild on rotting fruits. This environment provides them with a variety of benefits, such as protection from predatory wasps and parasites, nutrition, a positive teaching signal and increased likelihood of survival. However, high concentrations of ethanol are detrimental to the animal -although *Drosophila* can counteract some effects with inducible *Adh*-, resulting in death, decreased pupation rate and deformations.

This thesis addresses the question, whether such a crucial substance as ethanol, which functions as an odorant and has also intoxicating effects, can be used as a suitable reinforcer in either appetitive or aversive conditioning in *Drosophila* larvae by approaching the question systematically.

Indeed, this thesis was able to show that larvae are attracted to the smell of ethanol in a concentration dependent manner and that larvae prefer low doses of ethanol mixed in agarose as a crawling surface. Ethanol was also affecting attraction to other odorants in a concentration dependent manner. Furthermore, a link between ethanol and fructose was uncovered because the presence of fructose completely blocks attraction to ethanol and some odorants that are also key odorants for fermentation processes. This thesis also sheds light on the observation, that larvae appear to be indifferent to low doses of ethanol, because the behavioural phenotype after conditioning was comparable to conditioning without reinforcement. Additionally, novel odorants during test always elicited an attraction behaviour, independent of whether flies would otherwise approach the known odorant or avoid it, therefore showing that flies can still distinguish between an odorant and the same odorant mixed with ethanol. Taken together, this thesis provides an extensive glance into the role of ethanol as a reinforcer. As expected, the relationship between ethanol and the larvae appears to be quite complex. The context, in which the ethanol is encountered significantly affects whether a larva perceives ethanol as something rewarding. Most likely, ethanol might primarily function as an olfactory guide to a carbohydrate food source and otherwise be not attractive to the animal. This provides a basis from which the role of ethanol for larval behaviour can be studied further.

Drosophila melanogaster $T\beta h^{nM18}$ mutant is primarily accepted to have a defect in the formation of appetitive and aversive short-term memory and aversive middle-term

memory, with long-term memory formation being intact. Additionally, *Tβh*^{nM18} mutants show changes in their metabolic traits.

This thesis addresses the question, whether *Tβh*^{nM18} mutant flies are indeed defective for appetitive short-term memory formation or whether the internal state of the fly has a strong effect on appetitive behaviour, therefore mimicking a STM defect.

Indeed, *Tβh*^{nM18} mutants were able to show appetitive short-term memory when the internal state was considered. *Tβh*^{nM18} show aversion after conditioning with 0.15 M sucrose and show attraction that is indifferent from genetic control following increased starvation or with yeast reinforcement. Virgin female *Tβh*^{nM18} flies show low, but significant appetitive short-term memory with 2 M sucrose, while male *Tβh*^{nM18} flies are forming long-term memory after short-term memory conditioning. Prolonged starvation shifts appetitive STM into LTM in *w*^{1118} and into rapidly consolidated ARM that is completely cold-shock resistant 2 minutes after training in *Tβh*^{nM18} flies. This thesis might be the first evidence of the existence of appetitive ARM formation in *Drosophila melanogaster* flies to date.

Starvation as the result of glycogen storage depletion also makes appetitive STM formation in *Tβh*^{nM18} mutant flies possible as well as blocking of internal state sensing by blocking of InR signalling onto octopaminergic neurons. Artificially altering glycogen levels in flight muscles and fat body resulted in appetitive STM in *Tβh*^{nM18} flies. Additionally, the function of octopamine as a switch in learning and memory formation was uncovered, where octopamine pulses at certain time points result in blocking of LTM as well as STM, while epinastine, an OA antagonist, revealed that when OA signalling is lacking, memory is shuttled to LTM after STM conditioning.

Taken together, this thesis uncovers a novel role for octopamine as a learning and memory switch and that *Tβh*^{nM18} mutants are indeed capable of forming appetitive short-term memory, with the proper reinforcer or internal state, but form exclusively appetitive LTM under high glycogen storage conditions, which is probably regulated by insulin-like signalling onto octopaminergic neurons.

Furthermore, the existence of a proper appetitive ARM was shown in *Tβh*^{nM18} mutants, suggesting an inhibitory role for this form of memory by octopamine. This thesis provides novel insight into the regulation of learning and memory through octopaminergic signalling and the role of the internal state in appetitive behaviour regulation.

Zusammenfassung

Drosophila-Larven finden Ethanol attraktiv, welchem sie in erster Linie in der Wildnis auf verrottenden Früchten begegnen. Hier zeigt sich das Leben auf Ethanol mit einer ganzen Reihe von Vorteilen: so z.B. Schutz vor Fressfeinden und Parasiten. Ethanol ist eine Quelle für Nährstoffe und ein positives Lernsignal und steigert die Überlebenswahrscheinlichkeit.

Gleichzeitig zeigen sich aber auch, vor allem bei hohen Konzentrationen, eine ganze Reihe von Nachteilen, obwohl *Drosophila* einige dieser Nachteile mit induzierbarer *Adh* kompensieren kann. Zu den Nachteilen zählen Tod, verringerte Verpuppungsrate und Entwicklungsstörungen.

Das Ziel dieser Thesis war es zu analysieren, ob eine Substanz wie Ethanol -die große, physiologische Bedeutung für *Drosophila*-Larven hat- auch als Verstärker für olfaktorische Konditionierung benutzt werden kann. Die Thesis geht systematisch an die Fragestellung heran.

Tatsächlich zeigt die Thesis, dass Larven dosisabhängig attraktiv auf Ethanol als Duftstoff reagieren und, dass sie geringe Konzentrationen von Alkohol bevorzugen, um sich darin aufzuhalten. Ethanol zeigte einen deutlichen, dosisabhängigen Einfluss auf die Präferenz der Larven von anderen Duftstoffen. Zusätzlich offenbarte sich ein Zusammenhang zwischen Ethanol und Fruktose, da die Anwesenheit von Fruktose die larvale Duftpräferenz für Ethanol und einiger Schlüsseldüfte im Zusammenhang mit Fermentationsprozessen, komplett negierte.

Weiterhin zeigte sich während der Thesis, dass Larven auf geringe Ethanol Konzentrationen offenbar neutral reagieren, da sie nach Konditionierungsversuchen ein Verhalten zeigten, welches sich auch in Konditionierungsexperimenten die komplett ohne Verstärker auskamen, wiederfinden ließ.

Zusätzlich zeigten Larven eine konsequente und signifikante Präferenz für unbekannte Düfte, die während des Tests präsentiert wurden. Selbst dann, wenn der zweite, gepaarte Duft, eigentlich sonst immer Attraktionsverhalten ausgelöst hatte. Dadurch zeigt sich, dass Larven zwischen einem Duftstoff und demselben Duftstoff in einem Mix aus Duft und Ethanol unterscheiden können.

Zusammengenommen zeigte die Thesis einen ausgiebigen Blick auf die Rolle von Ethanol als Verstärker in Konditionierungsexperimenten. Wie erwartet, ist die Beziehung zwischen der Larve und dem Ethanol-Verstärker kompliziert und nicht so

leicht zu durchdringen. Der Kontext scheint dabei eine entscheidende Rolle zu spielen. Welche Dosis, zu welchem Zeitpunkt die Larve bekommt, entscheidet darüber, ob der Verstärker überhaupt als solcher wahrgenommen wird.
Denn, in erster Linie erscheint es so, als würde Ethanol hauptsächlich als Duftstoff für die Präsenz von Kohlehydraten funktionieren. Auf dieser Basis können weiterführende Experimente durchgeführt werden, um das Verhältnis zwischen Ethanol und Larve noch tiefer zu durchdringen.

Die *Drosophila*-Mutante $T\beta h^{nM18}$ ist bekannt als Lernmutante für appetitives, sowie aversives olfaktorisches Kurzzeitgedächtnis und Mittelzeitgedächtnis. Langzeitgedächtnis scheint bei den Tieren intakt zu sein. Zusätzlich zeigt die Mutante Veränderungen in ihrem internen Zustand.
Die Thesis untersucht, ob es sich bei der $T\beta h^{nM18}$-Mutante wirklich um eine Lernmutante handelt, oder ob nicht der interne Nährstoffzustand des Tieres einen enormen Einfluss auf das Verhalten hat und das Tier nur so aussehen lässt, als handele es sich um eine Lernmutante.
Tatsächlich zeigte sich, dass $T\beta h^{nM18}$ sehr wohl in der Lage ist Kurzzeitgedächtnis auszubilden. Entscheidend ist dabei nur, dass man den internen Zustand des Tieres berücksichtigt. Männchen zeigen aversives Kurzzeitgedächtnis mit 0,15 M Saccharose als Verstärker. Zusätzlich haben unbefruchtete Weibchen ein deutliches appetitives Kurzzeitgedächtnis mit Zucker gezeigt, während Männchen auch mit Hefe als Verstärker deutliches Kurzzeitgedächtnis zeigen. Außerdem zeigen Männchen die Fähigkeit mit Saccharose als Verstärker direkt Langzeitgedächtnis zu formen, obwohl sie nur in einem Kurzzeitgedächtnisprotokoll trainiert wurden.
Wenn diese Tiere lange hungern, zeigt sich in der genetischen Kontrolle w^{1118} eine Verschiebung von dem Kurzzeitgedächtnis ins Langzeitgedächtnis nach Training mit Saccharose, während die $T\beta h^{nM18}$-Mutante appetitives ARM (Anästhesie-resistentes Gedächtnis) zeigt, welches bis dato noch in keiner anderen Publikation nachgewiesen werden konnte.
Hunger als ein Resultat aus künstlich herunterreguliertem Glykogenhaushalt führt ebenfalls zur Ausbildung von appetitivem Kurzzeitgedächtnis nach Saccharose-Konditionierung. Ebenso zeigte sich, dass appetitives Kurzzeitgedächtnis in $T\beta h^{nM18}$ möglich ist, wenn man den internen Energiesensor -Insulin- ausschaltet.

Darüber hinaus offenbart die Thesis eine neue Funktion von Octopamine in der Regulierung von Gedächtnisformen. Es scheint, dass Octopamine wie ein Schalter funktioniert, der Langzeitgedächtnis aber auch Kurzzeitgedächtnis blockieren kann, wenn octopaminerge Neurone zu bestimmten Zeitpunkten aktiv sind. Ebenso zeigte sich, dass die Blockierung von Octopamine durch Epinastine zu der Formierung von Langzeitgedächtnis in genetischen Kontrollfliegen führte.

Zusammengenommen zeigte sich, dass Octopamine wie ein Schalter funktioniert, der Lernen und Gedächtnisbildung reguliert und das $T\beta h^{nM18}$ sehr wohl in der Lage ist appetitives Kurzzeitgedächtnis zu formen, auch wenn die Mutante normalerweise nur Langzeitgedächtnis zu formen scheint. Außerdem zeigte sich, zum ersten Mal, die Existenz eines appetitiven AR-Gedächtnisses (ARM), was für eine inhibierende Rolle von Octopamine in der Regulation von Gedächtnis spricht.

Die Thesis bringt neue Erkenntnisse für das Verständnis der Regulation von Gedächtnis durch Octopamine und die Rolle des internen Nährstoffzustandes als Regulator von appetitivem Verhalten.

Introduction

Animal behaviour is a highly complex mechanism that is regulated and modified by both, internal and external cues. Modification of behaviour is a strategy to facilitate survival of animals and allows adaptation to an ever-changing environment. One such strategy is the internal-state monitoring (Friedman, 1997; Hardie et al., 2012; Mayer and Thomas, 1967), that allows the animals to properly adapt behavioural output, in case that energy demands rise, and internal storages have to be replenished.
Behaviours, such as food consumption and appetitive olfactory learning, are key appetitive behaviours that are crucial in enabling survival and are thoroughly studied. Many different approaches are used to study animal behavioural adaptation. *Drosophila melanogaster* is a suitable model organism to address questions regarding neurophysiological fundamentals of behavioural modification. In this thesis *Drosophila melanogaster* are used in two different stages of their life cycle. On the one hand L3 larvae are used to address the question of rewarding properties of ethanol in a conditioning set up. On the other hand, adult flies are used to address octopaminergic and internal-state driven regulation of both feeding and appetitive olfactory learning. In all three main experimental approaches the ability of the animal to smell certain environmental stimuli is used, which is why it is of interest to first address how smell is perceived in these animals.

The *Drosophila* olfactory system and odour processing

Smelling is a key component to help animals to orientate in a chemosensory enriched environment. The basic structure of any organism that is capable of smelling anything in their surrounding are the odorant receptors (OR) (Vosshall and Stocker, 2007). Genes that encode for the ORs were found in complex organisms such as rodents (Buck and Axel, 1991), but also in simpler organisms such as *Caenorhabditis elegans* (Sengupta et al., 1996) or *Drosophila melanogaster* (Vosshall et al., 1999).
Both, larvae, and adult *Drosophila* express ORs on olfactory receptor neurons. These neurons project from the dorsal organ in the larvae (Vosshall and Stocker, 2007) and the maxillary palps and the antennae in adult *Drosophila* (Hallem et al., 2004; Shanbhag et al., 1999). The ORNs projection terminates in the antennal lobe. Here, distinct ORNs project towards distinct glomeruli (Ramaekers et al., 2005). While only

21 ORNs project onto glomeruli in larvae, the adult *Drosophila* has 1300 ORNs (Ramaekers et al., 2005). Glomeruli in the antennal lobe are not only projecting to higher brain regions, such as the mushroom bodies and the lateral horn, but they also have local interneurons (Ng et al., 2002; Ramaekers et al., 2005). Those interneurons are present in larvae and adults, some of which persist through the metamorphosis to the adult fly (Liou et al., 2018). They can be both excitatory and inhibitory and thus contribute to a sophisticated olfactory computation, that drives behaviour (Huang et al., 2010; Nagel and Wilson, 2016; Ng et al., 2002; Shang et al., 2007). A schematic overview of the *Drosophila* wiring diagram can be seen in Figure 1.

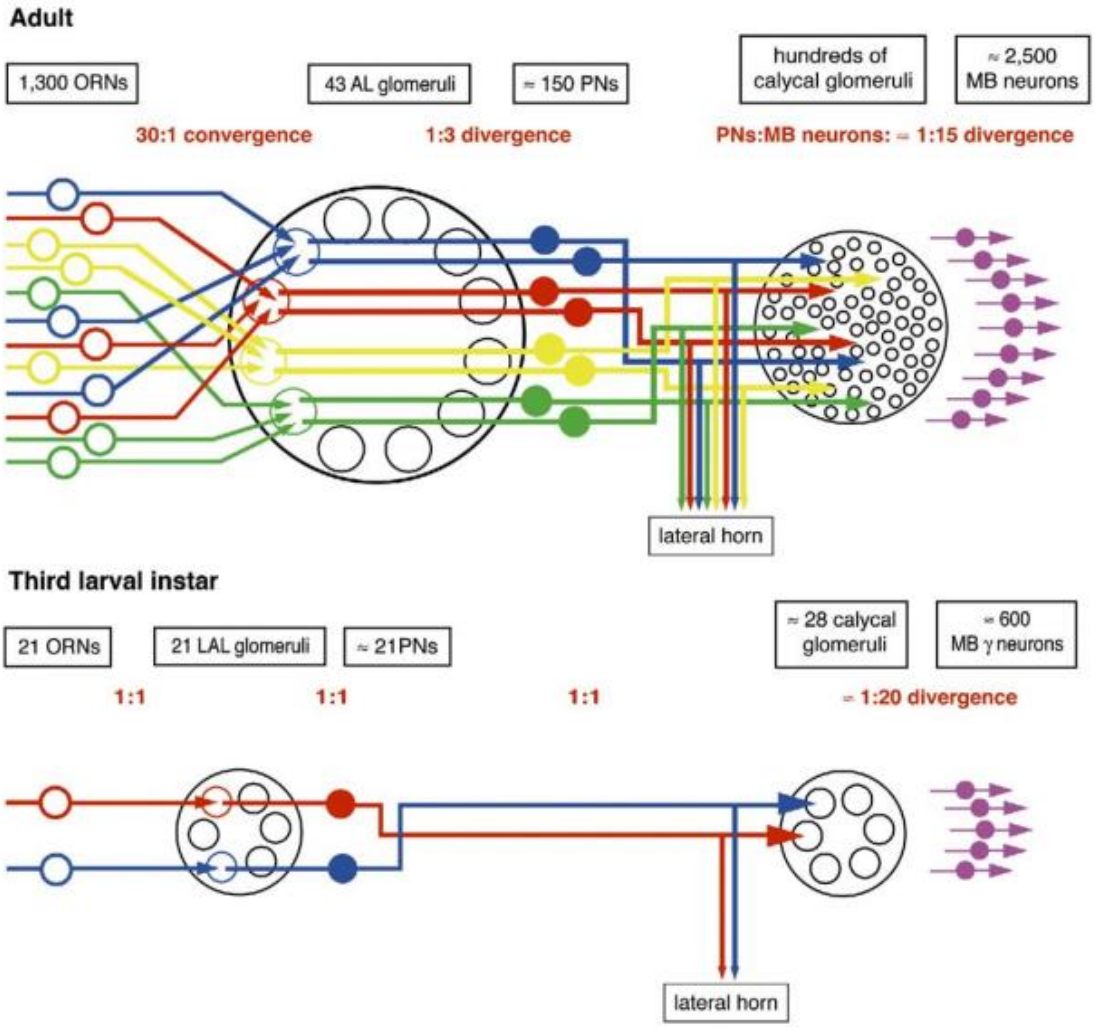

Figure 1: Schematic overview of the olfactory wiring of *Drosophila melanogaster* larvae and adults. Figure taken from Ramaekers et al.,2005.

An odour in nature is a volatile substance that is normally a mixture of different individual odorants. Overripe fruits for example, that are highly attractive to *Drosophila,* consist of a broad variety of individual volatiles (Zhu et al., 2003). These bouquets can be smelled by the fruit fly and their larvae and it is suggested, that the different components of the blend are responsible for the response of the animal (Silbering and Galizia, 2007). Not only the mixture but also the concentration of an odorant elicits different responses on the level of the antennal lobe as well as the projection neurons (Silbering and Galizia, 2007). Some odorants also result in inhibition of basal neuronal firing, which can both trigger aversion and attraction (De Bruyne et al., 2001; Cao et al., 2017). One such example is the *Or85a* expressing neuron, which in constitutively active and stops firing, when acetophenone binds to it (Cao et al., 2017). In general, it is accepted that specific receptors bind specific odorants (Vosshall et al., 2000). However, there are receptors that bind more than one type of odorant as well as odorants that bind to multiple receptors (Abuin et al., 2011). Most odours are attractive to the larvae (Fishilevich et al., 2005; Hoare et al., 2011; Kreher et al., 2008), even highly concentrated ones. Adult *Drosophila* avoid high concentrations of odorants (Jouandet and Gallio, 2015) and react with an alerted state, when they encounter novel odorants (Hattori et al., 2017). Odours in nature are signifiers of food (Engel and Tressl, 1983; Jirovetz et al., 2003) or oviposition sites (Quan and Eisen, 2018; Vesterberg et al., 2021). One such odorant, ethyl acetate, signifies the presence of yeast, which larvae consume and where adult *Drosophila* lay their eggs in (Giang et al., 2017; Yang, 2018). Odorants such as ethyl acetate are key odorants with innate meaning to the fruit fly (Giang et al., 2017). Chemical substances, such as ethanol, can be smelled and carry innate meaning to *Drosophila* both as a food source and as breeding ground (Geer et al., 1985; Giang et al., 2017; Lynch et al., 2017; McKenzie and Parsons, 1972; Parsons, 1980; Rao and Stokes, 1953). Therefore, a closer look at the relationship between ethanol and *Drosophila* is warranted.

Ethanol and the fruit fly

In nature, ethanol is an important olfactory cue. It signifies the presence of rotten fruit (Geer et al., 1985; Giang et al., 2017; Lynch et al., 2017; Parsons, 1980; Rao and Stokes, 1953). Ethanol is attractive to the larvae (Khurana and Siddiqi, 2013) and adult *Drosphila* (Xu et al., 2016). Although, ethanol is commonly accepted to be attractive to

Drosophila and play a huge ecological role (Giang et al., 2017; Gibson and Wilks, 1988; Gibson et al., 1981; Xu et al., 2016) it is surprising that the exact receptor to perceive ethanol is still unknown, with Or22a being a potential candidate (Hallem and Carlson, 2006; Mansourian and Stensmyr, 2015).

Still, environmental ethanol has many positive effects on and for *Drosophila*. It was shown to provide protection from predatory wasps, it is a source of nutrition and a suitable and very attractive breeding ground for adult *Drosophila* (Geer et al., 1985, 1989; McKenzie and Parsons, 1972; Milan et al., 2012; Richmond and Gerking, 1978; Schumann et al., 2021). For adult *Drosophila* ethanol is also an attractive substance on which they can feed. Although, they choose other substances, when they offer equal or better caloric value (Pohl et al., 2012). Interestingly, larvae appear to crawl out of ethanol patches, in which the eggs develop, as if they try to avoid higher concentrations of ethanol (Sumethasorn and Turner, 2016). This is an important observation, because -as with many things in nature- it is not always as simple as it seems.

The relationship towards ethanol is not only beneficial to *Drosophila*. To paraphrase Paracelsus: “The right dosage differentiates a poison from a remedy.” This is nowhere as true as it is with ethanol.

Ethanol is not only nutritious, it is also a neurotoxin (McClure et al., 2011). Growing up on high ethanol concentrations or ingesting too much of highly concentrated ethanol can have severe detrimental effects, such as neuronal changes, developmental delays or appendage abnormalities (Geer et al., 1988; Knabbe et al., 2022; McClure et al., 2011; McKenzie and Parsons, 1972; Ranganathan et al., 1987). Luckily to *Drosophila*, ethanol concentrations in nature rarely exceed 10 % (Gibson and Wilks, 1988; Gibson et al., 1981) and even then, it is mostly found in seepages in wineries, that specialise on fortified wine.

Larvae and *Drosophila* can compensate for some of the negative effects of ethanol by inducible *Adh*, which can be upregulated, if necessary (McKechnie and Geer, 1984). Adh is the alcohol dehydrogenase, which is responsible for up to 90 % of ethanol degradation in the fruit fly (Geer et al., 1985). The rest is degraded by other enzymes (e.g. catalase) (Geer et al., 1993). Ethanol is degraded into acetaldehyde first, by Adh, and in a second step to acetate, by Aldh -aldehyde dehydrogenase (Sha et al., 2014). When *Adh* is defect, or lacking -in mutants-, larvae show a significantly reduced survival rate and develop less likely into adult fruit flies (Heinstra et al., 1987). Furthermore, they show significantly reduced aversion to high concentrations of

ethanol (23%) and reduced preference to low doses of ethanol (5%) (Ogueta et al., 2010). Although Aldh is also an important enzyme in the degradation and thus detoxification of ethanol, it is not as intensely studied as Adh. Aldh is indeed important in ethanol tolerance in both larvae and adults, as was shown with *Aldh*-null mutants, that show significantly reduced tolerance to low ethanol concentrations that were tolerated by wild-type animals (e.g. 2 %) (Fry and Saweikis, 2006).

Additionally to Adh and Aldh as an important enzyme to regulate ethanol degradation and preference, neuropeptides, such as Corazonin, were reported to regulate ethanol-related behaviour and metabolism as well (Sha et al., 2014). Corazonin and its receptor appear to play a role in recovery from ethanol-induced sedation (Sha et al., 2014). Surprisingly, not much is known about ethanol as a potential reinforcer in olfactory conditioning, with only a few publications investigating ethanol as a rewarding (or punishing) reinforcer (Nunez et al., 2018; Petruccelli et al., 2018; Schumann et al., 2021). This was one of the topics addressed during this thesis and for this a closer look at olfactory learning in larvae and adult *Drosophila* is necessary.

Olfactory learning and memory in *Drosophila*

Learning is one of the most crucial abilities of any animal to maximise survival probability in a constantly changing environment. In simple model organisms, such as *Drosophila melanogaster*, learning can be observed in the form of olfactory conditioning, where the inherent meaning of an odorant (or ideally a completely neutral stimulus), be it aversive or attractive, can be reprogrammed by pairing it to a reinforcer that elicits either aversion or attraction (Heisenberg et al., 1985; Quinn and Dudai, 1976; Scherer et al., 2003; Tempel et al., 1983; Tully and Quinn, 1985). This conditioning works in both larvae and adult *Drosophila*. These animals are capable of learning to associate an odorant with a reward (sucrose, or fructose mostly) or a punishment (quinine, electric shock, or salt mostly). An odorant, the conditioned stimulus (CS), can be paired with either a reward or punishment -the unconditioned stimulus (US) and will thus become the CS+. A second odorant (CS) will be presented to the animal and will not be paired with reward or punishment and thus becomes the CS-. During the test larvae or adult *Drosophila* will get the chance to choose between both previously presented odorants, without the presence of the US and will show either approach or avoidance. This form of conditioning is called classical conditioning

and is based on observations made by Pavlov many years ago, where he was able to couple the ringing of a bell with the promise of food, which resulted in behavioural adaptation in a dog (Pavlov, 1927). After learning, that the ringing of the bell was followed up with food presentation, the animal anticipated the food and started salivating, even when no food was offered afterwards. Over the years many different versions of this simple -yet effective- experiment were developed and performed to uncover surprising and interesting things about learning and especially about the molecular basis of learning behaviour (Das et al., 2014; Eschment et al., 2020; Huetteroth et al., 2015; Knabbe et al., 2022; Michels et al., 2017; Nunez et al., 2018; Petruccelli et al., 2018; Schumann et al., 2021; Tempel et al., 1983; Tully et al., 1994; Tully and Quinn, 1985; Weiglein et al., 2019; Widmann et al., 2016).

When an animal changes its behaviour following experience, it is called learning. When learning persists over a certain amount of time, especially after repetition, it is called memory.

The *Drosophila* mushroom bodies

One of the central brain information processing structures, that are crucial for olfactory learning and memory, are the mushroom bodies (Stopfer, 2014) seeing that chemical ablation of these resulted in learning deficits in *Drosophila* (De Belle and Heisenberg, 1994).

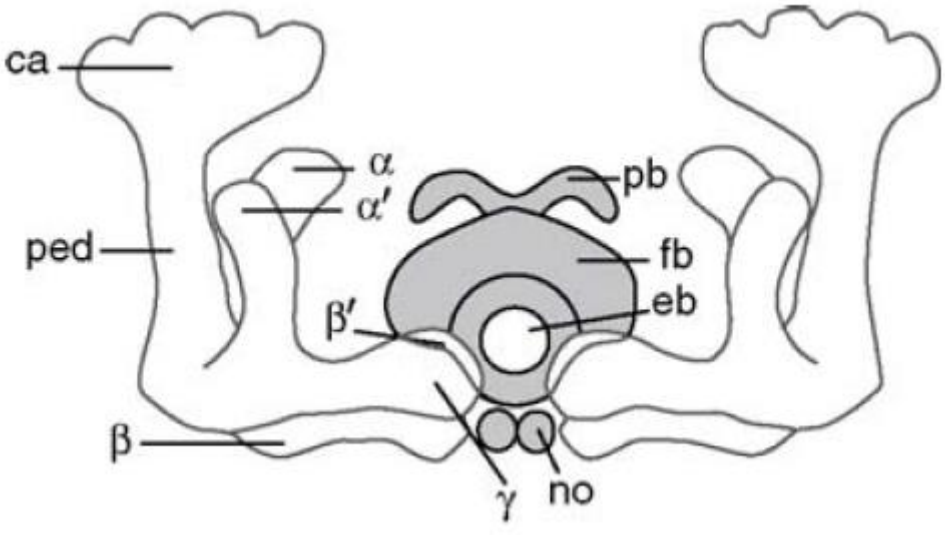

Figure 2: Schematic overview of the mushroom bodies and structures in proximity. Structures seen here are the mushroom body calyx (ca), the mushroom body peduncles (ped), the Alpha- (α) and

Alpha-prime lobes (α'), the Beta- (β) and Beta-prime-lobes (β'), the Gamma-lobes (γ), as well as the elipsoid body (eb), fan-shaped body (fb), the protocerebral bridge (pb) and the noduli (no). Modified after Kahsai and Zars, 2011.

The distinct mushroom body structure is made up of Kenyon cells (KCs), which form the basis of the different lobes, as described in Figure 2 (Stopfer, 2014). All kinds of neurons innervate the mushroom bodies. They are called mushroom body input neurons (MBINs), while neurons which project away from the mushroom bodies are called mushroom body output neurons (MBONs) (Saumweber et al., 2018; Scaplen et al., 2021). It was long thought that dopaminergic neurons (DANs) innervating the mushroom bodies had aversive reinforcing properties, while octopaminergic neurons (OANs) were said to carry appetitive reinforcing properties (Schwaerzel et al., 2003). However, more recent publications were able to show that appetitive information is mediated via dopaminergic neurons of the protocerebral anterior medial (PAM) cluster (Liu et al., 2012).

Appetitive memory can be reinforced by two things: The sweet taste and the nutrient value of the offered reward (Burke and Waddell, 2011), both are mediated by different neuronal subsets. DANs that convey sweet taste information are known to be activated by octopamine through an α-adrenergic like OAMB receptor (Burke et al., 2012). In general, DANs are known to mediate different information about food such as bad taste, sweet taste, nutrient value and even water which was reviewed by Das, Lin and Waddell in 2016 (Das et al., 2016).

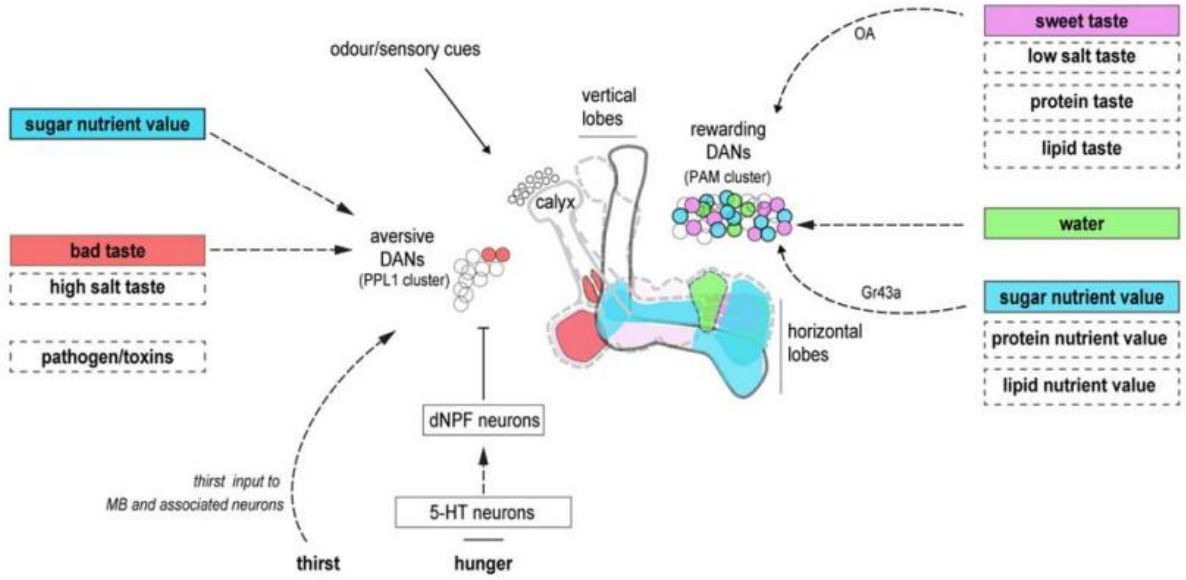

Figure 3: Schematic overview of the PAM and PPL1 cluster. Subsets of DANs that convey different properties of food reward to different lobes of the mushroom bodies. Taken from Das, Lin and Waddell, 2016.

The physiology of learning and memory formation

In general, olfactory associative memory forms when the Kenyon cells receive simultaneous information form neurons conveying CS information and US information. However, short-lived memory traces also form in the antennal lobe shortly after olfactory conditioning (Yu et al., 2004). Coinciding signalling through cholinergic and dopaminergic neurons, the first conveying CS information while the latter conveys US information, results in upregulation of cyclic adenosine monophosphate (cAMP) in the cell, which in turn binds to protein kinase A (PKA). Conformational changes reveal the catalytic unit of the PKA and the PKA starts to phosphorylate its substrates (Kandel, 2012; Widmann et al., 2016) (Figure 3).

In general, memory can either be short-lived with low levels of synaptic plasticity, or long-lived, where immense changes to the synaptic plasticity happen. When neurotransmitter stimulation is repeated several times the levels of cAMP increase for a longer period of time, which results in PKA recruiting p43 MAPK and are translocated to the nucleus of the cell (Davis, 2011; Kandel, 2012) where the cAMP response element binding protein (CREB), a transcription factor, is phosphorylated to induce long-term memory formation.

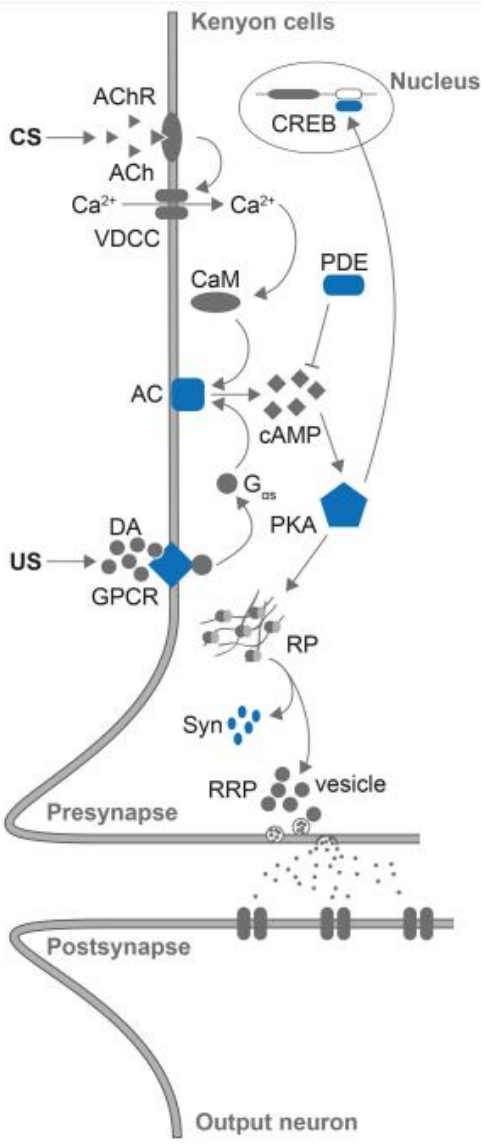

Figure 3: Molecular mechanism of olfactory learning in the Kenyon cells of the mushroom bodies. CS information is conveyed through cholinergic neurons, which recruit CaM, which in turn upregulates cAMP synthesis through the adenylyl cyclase (AC). Simultaneously, the US information is conveyed through DA-MBONs which leads to dissociation of the G-protein form the G-protein coupled receptor. Both, the G-protein, and CaM activate the AC as a result of coincident stimulation, which leads to increased levels of cAMP, a second messenger. The phosphodiesterase (e.g., dunce) (PDE) negatively regulates cAMP levels. cAMP activates protein kinase A (PKA), which either phosphorylates Synapsin or CREB, depending on whether short-term memory or long-term memory is formed. Modified after Widmann et al. 2016.

Forms of memory in *Drosophila*

Memory can take many different forms, mostly distinguishable through its retention time. In general, the scientific community distinguishes short-term memory (STM), middle-term memory (MTM), long-term memory (LTM) as well as anaesthesia-

resistant-memory (ARM) and anaesthesia-sensitive memory (ASM), which was reviewed by Margulies and Tully in 2005 (Margulies et al., 2005) (Figure 4).

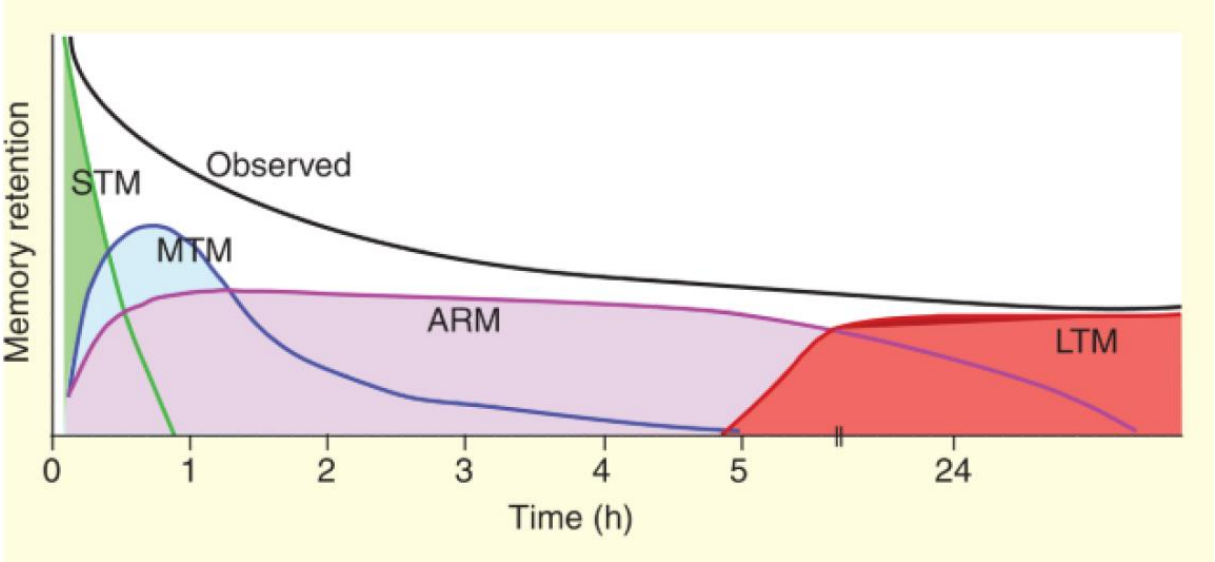

Figure 4: Schematic overview over different forms of memory that can be distinguished in *Drosophila melanogaster.* Short-term memory (STM) forms primarily after one-cycle of conditioning and lasts for approximately an hour. Middle-term memory (also called intermediate-memory (Murakami et al., 2017)) (MTM/ITM) lasts for a longer period and requires *amnesiac* to function properly. Long-term memory must be consolidated and last for several days. It requires de novo protein-synthesis and CREB activity in the nucleus. A special form of memory is anaesthesia-resistant memory, which does not have to be consolidated and requires *radish* function and is protein-synthesis independent. Taken from Margulies and Tully, 2005.

In *Drosophila* one trial of olfactory conditioning results in labile short-term memory, that normally lasts for approximately one hour (Tempel et al., 1983; Tully and Quinn, 1985). This memory consists of anaesthesia-sensitive memory, which depends on Synapsin (Knapek et al., 2010; Tully et al., 1994) and is almost completely sensitive to cold-shock anaesthesia. However, repetition of trainings cycles and time between training and test affect the amount of anaesthesia-resistant memory that forms (Bourouliti and Skoulakis, 2022; Tully et al., 1994) and probably also shifts memory from short-term memory into middle-term memory. The amount of shock-pulses given during short-term memory conditioning -which normally consist of 12 short pulses over 1 minute with intermediate breaks- also affects how high the observed learning index will be (Tully and Quinn, 1985). Another form of memory, that forms after one-cycle of conditioning, is the middle-term memory (MTM/ITM), which consists of anaesthesia-sensitive and anaesthesia-resistant memory (Murakami et al., 2017). This memory

requires *amnesiac* function (Tully and Quinn, 1985; Turrel et al., 2020), seeing that *amnesiac* mutants show significantly lower levels of STM directly after training and have a rapid decay of memory (Tully and Quinn, 1985), although the low memory scores then persist for at least 7 hours.

To form more robust forms of memory, the trainings protocol must be adapted. Two classical ways of adapting are frequently used in olfactory conditioning experiments. Massed training and spaced training. The first being multiple conditioning cycles that are performed in rapid succession without intermediate breaks. The latter requires breaks of 15 minutes in-between conditioning cycles. Training is performed at least 5 and often up to 10 times (Bourouliti and Skoulakis, 2022; Tully et al., 1994). Massed training results in memory that last for up to three days and is independent of protein-synthesis, which experiments with cycloheximide (CXM) -a protein-synthesis inhibitor- uncovered (Margulies et al., 2005; Tully et al., 1994). Aversive memory following massed training is not erased by CXM treatment (Tully et al., 1994), while appetitive memory is still sensitive to CXM treatment (Colomb et al., 2009). This memory is cold-shock insensitive, hence the name anaesthesia-resistant memory (ARM) although a dependency on repetition was shown where 5 massed-training cycles still resulted in ARM that also consisted of anaesthesia-sensitive memory directly after training. Over time, levels of ARM increase (Bourouliti and Skoulakis, 2022; Margulies et al., 2005).

On the other hand long-term memory is cold-shock sensitive when the cold shock is applied directly after the training and only after consolidation LTM gets cold-shock insensitive (Krashes and Waddell, 2008; Tully et al., 1994). It requires *radish* and CREB function and leads to de novo protein-synthesis. However, LTM can be disrupted by application of CXM or by genetically block LTM specific neurons (*R15A04-Gal4* as used by Katrin Auweiler as part of the work on the topic for this thesis) (Yamagata et al., 2015).

Feeding behaviour in *Drosophila*

Another crucial behaviour to ensure survival is the search for and the ingestion of nutritious food sources. For this, flies choose balanced diets for proper homeostasis of nutrients, even going so far as to cannibalize each other (or carcasses) in case of long starvation periods (Ahmad et al., 2015; Yapici et al., 2014). However, not only starvation has an influence on feeding choice, but also the taste of the food and the

ability to properly sense the nutritional value of a food source (e.g. *cupcake* mutants that lack a sodium/solute con-transporter-like protein and can no longer distinguish between nutritious and non-nutritious sugars), as was reviewed by Yapici in 2014 (Yapici et al., 2014).

Drosophila uses the olfactory system to track food sources (Giang et al., 2017; Zhu et al., 2003). Once a food source is located, the food source is ingested through the proboscis. Gustatory receptors (GRs), expressed on gustatory receptor neurons (GRNs) are used to taste the food source. GRs can detect sweet and bitter substances, while ionotropic receptors are necessary for amines and salt detection (Scott, 2018). Water is sensed by PKK channels (Cameron et al., 2010; Chen et al., 2010).

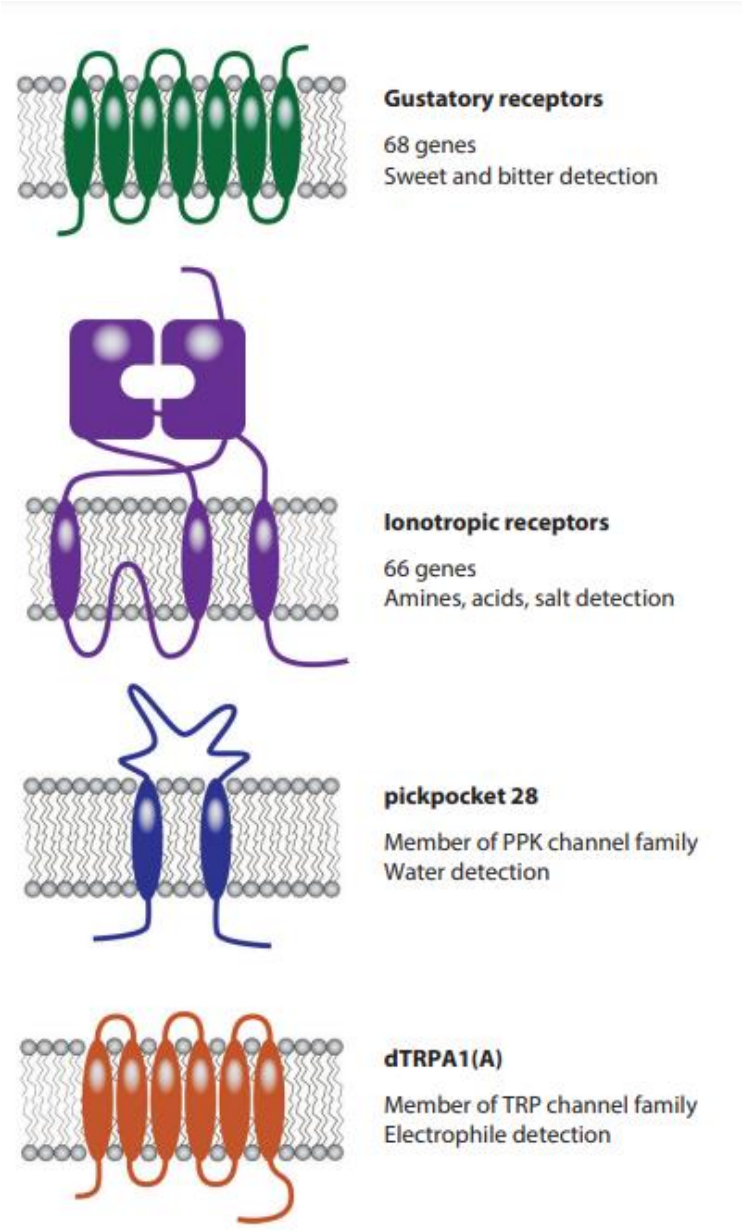

Figure 5: Schematic overview over the major taste receptor families and their respective functions in *Drosophila melanogaster.* Modified after Scott, 2018.

Gustatory receptors can be found all over the body surface (e.g the proboscis, the legs, the wings and -in female flies- on the ovipositor) (Scott, 2018). This way flies can sample food sources, without the need of ingesting it and -potentially- poison themselves (Scott, 2018).

When a fly tastes sugar with the legs, it stops movement, and the proboscis is extended. Sweet substance sensing results in ingestion, while bitter substances lead to proboscis retraction, as described nicely in the review by Kirsten Scott, 2018 (Scott, 2018). How long a fly consumes food and when it stops is determined by factors such as hunger and sugar concentration (Yapici et al., 2016). This is regulated by cholinergic interneurons, where IN1 neurons are needed for the regulation of sucrose ingestion. Silencing resulted in significantly reduced ingestion of sucrose, independent of the starvation state (Yapici et al., 2016).

Interestingly, the concentration of the food source also regulates how much a fly ingests. Low concentrations of sugar as a food source results in significantly less ingested food, although the fly constantly ingests during the time, while higher sucrose concentrations are ingested in higher amounts and also primarily at the beginning of feeding initiation (Yapici et al., 2016).

Sensitivity to sugar and the amount of PER (proboscis extension reflex) bouts increases with increased starvation as well as through dopaminergic signalling onto gustatory neurons, which express the Gr5a receptor and the DopEcR dopaminergic receptor on the same GRNs (Inagaki et al., 2012). Other neurotransmitters, such as octopamine, lead to depotentiation of the bitter taste through starvation induced reduction of firing rate in OA-VL neurons (LeDue et al., 2016). Octopamine is also known to regulate starvation induced hyperactivity, which is said to increase chance of food encounter (Yang et al., 2015). When food is encountered, locomotor activity significantly reduces and starvation-induced hyperactivity is suppressed (Betley et al., 2015; Chen et al., 2015; Yu et al., 2016). This starvation-induced hyperactivity is regulated by AKHR, a receptor for the adipokinetic hormone (AKH) -a glucagon homolog in *Drosophila*- and insulin-like signalling onto AKHR-positive neurons (Yu et al., 2016). While AKH signalling promotes hyperactivity, co-expressed InR indeed inhibits hyperactivity. Interestingly, the circuits for sugar sensitivity through the G5a

receptor and starvation-induced AKHR-mediated hyperactivity are two independent circuits, that both promote the finding and ingestion of nutritious food (Yu et al., 2016). Satiety is promoted through insulin-producing cells (IPCs) in the brain, which appear to be innervated by DTK-positive neurons, where *Drosophila* tackyinin (DTK) binds to TAKR99D, the DTK receptor. This way TAKR99D neurons regulate *Drosophila* insulin-like peptide (DILPs) release (Qi et al., 2021) and suppress feeding. DTK expression is upregulated in fed flies, this way promoting satiety.
Both, octopamine and insulin-like signalling are important parts of the second half of this thesis and therefore are in need to be properly addressed in a separate chapter.

Octopamine and behavioural regulation in Drosophila

Octopamine (OA) is reported to be the homolog to the mammalian adrenalin and noradrenalin (Roeder, 2020) and is thoroughly studied to this date. It is primarily known to be involved in the regulation of the internal-state as well as memory formation (Das et al., 2014; Huetteroth et al., 2015; Iliadi et al., 2017; Scheiner et al., 2014; Schwaerzel et al., 2003; Yang et al., 2015). Octopamines' role in memory formation has been shown in insects such as honey bees and the oriental fruit fly, *Bactrocera dorsalis* (Hammer, 1993; Yu et al., 2022).

Octopamine in learning

The $T\beta h^{nM18}$ is a mutant that was characterised as a null-mutant, unable to synthesis OA (Monastirioti et al., 1996). Flies that lack octopamine have shown to significantly lack the ability to form appetitive short-term memory as well as aversive short-term memory (Das et al., 2014; Iliadi et al., 2017; Sabandal et al., 2020; Schwaerzel et al., 2003). Furthermore, octopamine also regulates the formation of proper aversive anaesthesia-resistant memories (Wu et al., 2013) through APL neurons whose neuronal activity is necessary after conditioning.
Octopamine signalling downstream of *rutabaga* activity on the Oct1β receptor drives appetitive and aversive memory (Sabandal et al., 2020). The type of neuron determines whether it is aversive or appetitive memory, that is regulated by OA. In αß MB neurons OA regulates aversive memory, while OA in the projection neurons (PNs) is necessary for appetitive memory (Sabandal et al., 2020). In the KCs, octopamine

was reported to act in concert with nicotine, resulting in increased calcium levels with a synergistic effect (Leyton et al., 2014), and thus potentially contributing to the processes leading to memory formation.

Octopamine in feeding and decision-making

Octopamine is an extremely pleiotropic transmitter, regulating a broad variety of function in the organism (Roeder, 2020) (Figure 6).

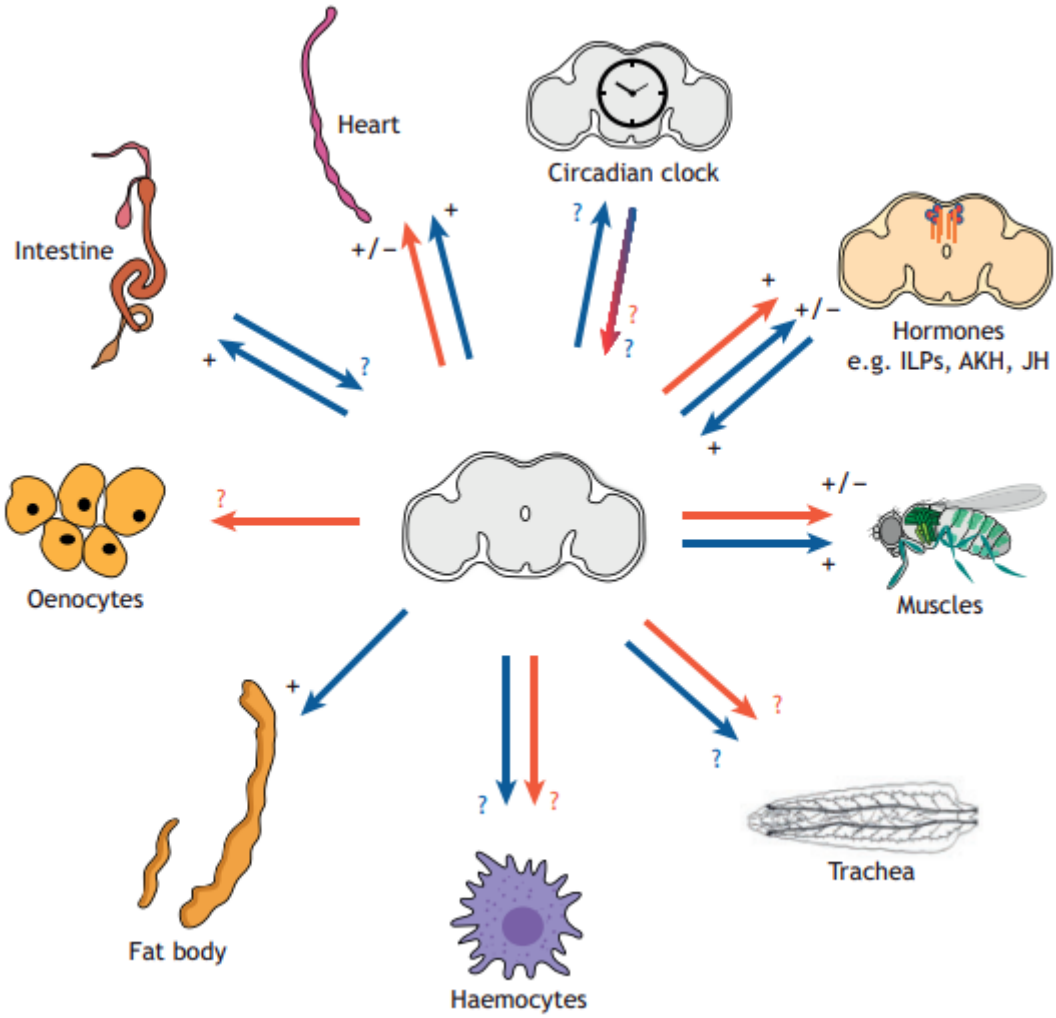

Figure 6: Schemtatic overview of the processes regarding metabolism regulated by OA and TA in *Drosophila*. OA (in blue) and TA (in red) regulate a broad variety of organs and their functions in the whole body, regarding the regulation of the internal state. A plus indicates positive regulation, while a minus indicates negative regulation. Question marks are an indicator for yet unclear functions. Taken from Roeder, 2020.

Starvation is known to trigger the release of octopamine in *C. elegans* (Tao et al., 2016). In *Drosophila,* starvation-induced hyperactivity is regulated by OA -among other things- which also links OA release to starvation (Yang et al., 2015). OA-VL neuronal activity is necessary to reduce the aversion to bitterness upon starvation, to ensure survival (LeDue et al., 2016). Starvation decreases activity in the OA-VL neurons, which normally potentiates bitterness aversion. Food intake appears to be regulated by OA as well, although here results differ between publications, making it hard to form a cohesive picture (Li et al., 2016; Roeder, 2020; Yang et al., 2015). What is indeed known to be regulated by OA, is the inhibition of constant food odour tracking. Activation of OA-VPM4 neurons results in suppression of odour tracking behaviour and promotes feeding initiation (Sayin et al., 2019; Youn et al., 2018). In *Drosophila* larvae octopamine was also shown as a regulator of food ingestion. In starved animals octopamine acts through VUM2-neurons to regulate ingestion, while simultaneously, through another circuit (VUM1-neurons) inhibiting appetite as a potential regulator to inhibit overeating (Zhang et al., 2013a).
Furthermore, OA is known to regulate decision making regarding the internal state. Mated females shift from carbohydrate food sources to protein-rich food sources, which is mediated by OA neurons as well (Tian and Wang, 2018). Additionally to this, OA was reported to function as a decision-making switch, depending on which neuronal subsets are activated (Claßen and Scholz, 2018).

Octopamine in metabolism

Octopamine is known to regulate metabolism as well. Flies lacking OA, e.g $T\beta h^{nM18}$ mutants, show significantly increased levels of fat, which mimics an obesity phenotypes in mammals (Li et al., 2016). The authors provide further inside into the matter. Thus, they report that flies lacking OA show lower food intake, while simultaneously showing lower energy level depletion probably due to decreased physical activity (Li et al., 2016).
Indeed, octopamine was reported to regulate climbing ability in fruit flies. Lacking OA, flies performed poorly in negative geotaxis assays (El-Kholy et al., 2022; Li et al., 2016). Therefore, the lower energy expenditure in OA-lacking flies can also be a motor control defect or lack of motivation, as reviewed by Selcho and Pauls, in 2019 (Selcho and Pauls, 2019).

Octopamine is known to stimulate the release of fructose-2,6-bisphopshate, which - with AMP- synergistically activates glycolysis, providing an insect with energy during flight (Pflüger and Duch, 2011; Wegener, 1996). Alas, these findings have yet to be confirmed in *Drosophila melanogaster* specifically.

Insulin-like signalling in *Drosophila*

A key player in monitoring the internal state in *Drosophila* are the insulin-like peptides (Nässel et al., 2015), which are secreted by IPCs in the brain and fat body.
8 insulin-like peptides are known in Drosophila which are expressed in different tissues and serve different functions in the fly organism (Brogiolo et al., 2001; Colombani et al., 2012). Among these functions are regulation of cell growth, organ- and body-size and cell proliferation.
On a behavioural level DILPs and signalling from and onto IPCs and are known to regulate social behaviour, such as aggression and courtship latency, feeding behaviour and associative aversive learning in larval and adult Drosophila (Chambers et al., 2015; Eschment et al., 2020; Luo et al., 2014; Naganos et al., 2012; Zandawala et al., 2018).
In larvae, InR expression on the KCs resulted in suppression of larval aversive ARM, when larvae were fed before aversive conditioning experiments. Insulin-like signalling thus functions as a gating mechanism between two forms of memory in larval *Drosophila* (Eschment et al., 2020). Insulin-like signalling is also necessary in the MBs in adult *Drosophila* for aversive olfactory learning, while memory retention requires insulin-signalling in the ellipsoid body (Chambers et al., 2015).
Cortex glia are known to provide glucose to neurons upon formation of olfactory LTM, which can be disrupted by InR signalling block in cortex glia (de Tredern et al., 2021). Furthermore, glucose shuttling to the MB neurons is downregulated, when InR signalling is blocked onto cortex glia (de Tredern et al., 2021).
Dilp3, secreted by IPCs in the *Drosophila* brain, is necessary for the formation of aversive MTM (Tanabe et al., 2017). This specific form of insulin-like signalling actually happens at the fat body of the adult *Drosophila* and is disrupted in aged flies (Tanabe et al., 2017).
Chico, one of the *Drosophila* insulin receptor substrates, is also involved in the formation of olfactory associative memory (Naganos et al., 2012). Chico is expressed

in the Kenyon cells and the mushroom bodies of *Drosophila. Chico* mutants show specifically defects in the formation of memory, not in the retention or retrieval of it (Naganos et al., 2012).

Surprisingly, all these studies focus primarily on aversive memory formation, retention and retrieval and the role DILPs or InR might play in it. Because insulin is strongly associated with energy homeostasis and food ingestion, this would suggest that the regulation of appetitive memory formation through insulin-like signalling would be thoroughly studied. It can only be speculated about, why it is not the case.

However, fact is that InR signalling affects *Drosophila* consumption behaviour. As already mentioned, IPCs receive DTK and TAKR99D signalling, which results in suppression of food ingestion during ongoing feeding (Qi et al., 2021). Another circuit, mediated by 5-HT neurons that signal onto IPCs, after detection of ingested carbohydrates, triggers decrease of food intake to reduce overconsumption (Yao and Scott, 2022). Interestingly, the same study also revealed a second 5-HT neuronal circuit, that is activated by bitter substance ingestion, which reduces ingestion and activates gut motility, probably to induce secretion of nutrients in a situation, where non can be taken in by food ingestion (Yao and Scott, 2022). Additionally, IPCs express OAMB, an octopamine receptor, as well as 5-HT_{1A}, a serotonin-receptor (Luo et al., 2014). They appear to have opposite functions. On the one hand, OAMB regulates *dilp3* secretion, which is necessary for aversive MTM formation (Tanabe et al., 2017). On the other hand, the 5-HT_{1A} receptor regulates *dilp2* and *dilp5* expression.

Different DILPs were also shown to influence protein-rich food source consumption. In *Dilp3, Dilp5* and *Dilp7* mutants an increase in consumption was observed for high concentration of yeast extract, which suggests that DILPs secretion negatively regulates protein-consumption (Semaniuk et al., 2018). The same mutants also showed differences in circulating levels of haemolymph sugars (Semaniuk et al., 2018). Interestingly, although the three D*ilp* mutants show increase in protein-consumption, D*ilp3* and D*ilp*7 show increased haemolymph sugar, while D*ilp5* shows downregulated haemolymph sugar.

A link between protein-sources and sugar, and that both substances affect the subsequent consumption behaviour, is known (Lebreton et al., 2014). Flies that starve before consumption experiments show significant increase in yeast consumption. Similarly, when flies are fed on sucralose, which is sweet but not nutritious, they consume more yeast during consumption experiments, while sugar consumption

significantly reduced yeast preference during experiments (Lebreton et al., 2014). This shift is mediated by proper InR signalling.
Insulin signalling on Drosulfakinin-producing cells, a neuropeptide involved in a variety of functions, has revealed a role in the regulation of starvation induced sleeplessness as well as reduced sleeping in fed flies (Palermo et al., 2022). Furthermore, the same study revealed an important role of insulin-like signalling onto Dsk (Drosulfakinin)-neurons in regulation of metabolic traits, such as triglycerides as well as food consumption (Palermo et al., 2022).
Taken together, insulin-like signalling is required for the regulation of aversive olfactory memory as well as satiety inducing behavioural changes in relation to the internal state. This is primarily regulated by signalling of different neurons onto IPCs to induce excretion of DILPs that, downstream, regulate consumption behaviour.

Scientific Aim

This thesis aimed at answering two questions for different life stages of *Drosophila melanogaster*. The first one being: Is ethanol a suitable reinforcer in larval olfactory conditioning and, if so, is it an aversive or attractive reinforcer?
Ethanol is known as a substance, that plays a crucial role in the life of a developing larva. The smell of ethanol provides clues about suitable oviposition sites, it provides protection against parasites, it can be ingested and used for lipogenesis and there are first hints that larvae might indeed find ethanol to be an attractive teaching signal in olfactory conditioning (Cavener, 1979; Geer et al., 1985; Gibson and Wilks, 1988; Gibson et al., 1981; Milan et al., 2012; Parsons, 1980; Richmond and Gerking, 1978; Schumann et al., 2021; Sumethasorn and Turner, 2016). However, ethanol is also a toxin and has negative effects on the developing larva, especially upon high concentrations (Fry, 2001; Geer et al., 1989; McClure et al., 2011). Therefore, a conclusive answer to this topic is yet to be determined.
Due to the different effects of ethanol, as an odorant as well as an intoxicating substance, and the fact that low concentrations of ethanol provide mostly beneficial, while high concentrations provide detrimental effects, the first step was to analyse how larvae react to different ethanol concentrations. Therefore, different concentrations were used (5, 8 and 10 %) in odorant cups to address, whether ethanol as an odorant was attractive to the larvae and which concentration was the most attractive.

Since ethanol smells, the odorant acuity to the odorants used in conditioning experiments had to be tested as well, to determine whether ethanol affected the attractiveness of odorants. Ethanol could also shift the balance of odorants, therefore odorant balance experiments were performed to analyse the behaviour of animals towards the CS, when it was mixed with ethanol. Additionally, it was of interest to address the question, whether ethanol as an odorant might affect the CS during conditioning and therefore be re-evaluated by the larvae as a potentially different odorant. This question was tackled with exchanging the CS+ or the CS- during test for a novel odorant and observe the behaviour of the animals.

To address the multiple physiological effects that ethanol has on the organism, the attraction or aversion to ethanol in agarose was tested, with plates that are ½ mixed with ethanol and ½ plain agarose.

The next step was to systematically address different olfactory conditioning paradigms with an attractive concentration of ethanol as a reinforcer, to uncover whether ethanol is attractive or aversive during conditioning. Conditioning without reinforcer addressed the question, whether repeated exposure to the CS during training could additionally affect the outcome of conditioning cycles.

Many different parameters must be adjusted to properly unravel the role of ethanol as a reinforcer. The concentration of the reinforcer, the odorants used during conditioning, the amount of training cycle (one-cycle or three-cycle) and the timepoint between training and test.

The next aspect, that had to be addressed, was how larvae would value ethanol. Larvae grow up in ethanol and ingest it, however in adult flies an isocaloric sucrose solution was shown to be significantly more attractive than ethanol (Pohl et al., 2012). For this, larvae were trained either on fructose plates completely, with ethanol functioning as a CS during conditioning, or they were trained on fructose and ethanol plates. This would address the question, whether they are more attracted to sugar than to ethanol during test on pure plates.

This part primarily focussed on a broad and systematic analysis of classical behavioural parameters and how ethanol presence during these experiments would alter the expected behavioural outcome.

The second question regarded adult *Drosophila* and the role of octopamine and the internal state in the regulation of appetitive behaviours. Specifically, consumption behaviour as well as learning and memory formation.

The $T\beta h^{nM18}$ mutant is known as a *Tβh* null-mutant and is therefore unable to synthesize octopamine (Monastirioti et al., 1996). This lack of octopamine was argued to result in appetitive as well as aversive learning defects (Das et al., 2014; Huetteroth et al., 2015; Iliadi et al., 2017; Schwaerzel et al., 2003), while one cycle of appetitive training still induced protein-synthesis dependent long-term memory (Krashes and Waddell, 2008). Furthermore, $T\beta h^{nM18}$ flies show dysregulated survival, haemolymph sugar, triglyceride levels and food consumption (Li et al., 2016; Roeder, 2020). However, whether $T\beta h^{nM18}$ is indeed a short-term learning deficit mutant, or may just -due to changes to the internal state- not show memory shortly after conditioning, is not properly investigated yet.

The first step was to address the ability of $T\beta h^{nM18}$ to form appetitive short-term memory. Therefore olfactory conditioning experiments were performed, following the basic protocol that was established by Tully and Quinn (Tully and Quinn, 1985), with either 0.15 M sucrose reinforcement or 2 M sucrose reinforcement. 0.15 M sucrose reinforcement was additionally chosen to address equal sucrose concentration as in the accompanying CAFE assays.

The internal state was measured through glycogen measurement, following the basic protocol of Tennessen et al (Tennessen et al., 2014). In flies, the internal state was first altered by simple starvation. Flies were either trained 16 h after starvation or 40 h after starvation, to see whether increased hunger might significantly alter memory formation in w^{1118} and $T\beta h^{nM18}$. To test, whether the hypothesis of the internal state as a regulator for appetitive memory in $T\beta h^{nM18}$ flies was valid, other internal states had to be considered during experimentation.

Therefore, female virgin and mated flies were trained with 2 M sucrose in a one-cycle appetitive conditioning experiment to address, whether they would form short-term memory. This way the known fact that mated flies switch to protein-rich food sources (Tian and Wang, 2018), could address the idea of behavioural changes towards sucrose as a reinforcer. Male w^{1118} and $T\beta h^{nM18}$ flies were also tested with yeast reinforcement during conditioning. A former lab member, Manuela Ruppert, performed Bradford tests (seen in Figure 28D) and found decreased total levels of protein in $T\beta h^{nM18}$ flies. Therefore, changing the reinforcer to yeast would also address the

question of alterations in the internal-state and how this affects appetitive short-term memory formation in *Tβh*^{nM18} flies.

The next step was to analyse what form of memory *w*^{1118} and *Tβh*^{nM18} form upon increased starvation. For this, three approaches were taken. First, flies were starved for either 16 h or 40 h, trained with 2 M and tested 6 hours after training. Approximately 5 hours after conditioning protein-synthesis dependent LTM can be observed after consolidation (Margulies et al., 2005). Second, to determine whether memory after 40 hours of starvation was protein-synthesis dependent LTM or cold-shock insensitive ARM, cold-shock experiments were performed. *w*^{1118} and *Tβh*^{nM18} flies were starved for 16 h or 40 h and cold-shocked 2 min or 2 h after training, to address the fact that cold-shock can erase LTM but not ARM when anaesthesia is applied shortly after conditioning (Eschment et al., 2020; Krashes and Waddell, 2008; Tully et al., 1994). Third, to address the fact that -at least- aversive ARM can also be erased by cold-shock, when the training was not repeated often enough or the cold-shock was given directly after training (Bourouliti and Skoulakis, 2022; Quinn and Dudai, 1976), a genetic approach was taken to specifically block only LTM formation in *Drosophila*.

For this third approach *R15A04-Gal4,* an appetitive LTM specific line that expresses Gal4 in dopaminergic appetitive LTM neurons (Yamagata et al., 2015), was crossed into the *Tβh*^{nM18} mutant and the R15A04 neurons were silenced with *UAS-shibire*^{ts} expression. The experiments were carried out by Katrin Auweiler, a bachelor student under my supervision. This way only appetitive LTM formation was blocked during the consolidation phase after conditioning and the question could be answered from another perspective.

To address starvation as a regulator of appetitive memory, artificially altering glycogen storages in major storage organs -the fat body and the flight muscles (Wigglesworth, 1949)- had to be performed. The Gal4-line *mef2-Gal4* and *FB-Gal4* were used, to address either flight muscle and brain (Crittenden et al., 2018) or fat body respectively. *Mb247-Gal80* was used as a proof of concept to analyse whether Gal4 expression in the mushroom bodies would affect consumption behaviour in the *Tβh*^{nM18} background. Glycogen synthase (*GlyS*) and glycogen phosphatase (*GlyP*) expression was knocked down with *GlyS-RNAi* and *GlyP-RNAi* (Yamada et al., 2018), to down- or upregulate glycogen storage in the organs respectively. A fly-line was crossed that carried the *mef2-Gal4* and *FB-Gal4* transgene simultaneously to address down- and upregulation

in both organs at the same time. Flies were tested in a short-term memory paradigm, to analyse whether changes to the glycogen storage would affect memory formation.

To address the question of glycogen storage being responsible for changes in appetitive learning in *Tβh^{nM18}*, *GlyS* was knocked down with RNAi in fat body, flight muscle or simultaneously in both storage organs and flies were trained and tested in a short-term memory paradigm. As a proof of concept that the genetic tools were properly functioning, PAS-staining assays were performed on larvae (Yamada et al., 2018, 2019).

To further investigate the role of the internal-state onto appetitive memory performance in *Tβh^{nM18}* flies, the major internal state sensing molecule, the *Drosophila* insulin-like peptide, (Brogiolo et al., 2001; Colombani et al., 2012; Nässel et al., 2015) had to be addressed.

For this dominant negative and constitutively active insulin-like receptors were expressed under the control of *Tdc2-Gal4* to specifically express the transgenes in octopaminergic neurons. Short-term memory conditioning and testing was performed in *w^{1118}* and *Tβh^{nM18}* background to analyse memory formation and CAFE assays were performed to investigate changes in consumption behaviour.

Lastly, to specifically analyse the role of octopamine in the formation of appetitive memory, short-term memory conditioning experiments were performed in *w^{1118}* and *Tβh^{nM18}* flies, which were fed with octopamine or epinastine, an OA antagonist, in a concentration that was used already to inhibit TfAP-2-induced hyperactivity in *Drosophila* (Claßen and Scholz, 2018; Williams et al., 2014).

To address LTM formation specifically, *Tβh^{nM18}* flies were fed OA and tested 6 hours after conditioning. It was already shown prior to this, that OA feeding in *Tβh^{nM18}* resulted in appetitive short-term memory 2 minutes after training (Schwaerzel et al., 2003). This new approach could show whether OA would also block LTM formation in *Tβh^{nM18}* flies. Epinastine was fed at different time points to *w^{1118}* to either block short-term memory formation or induce long-term memory formation in *w^{1118}* flies. This way octopamine as a switch in learning and memory formation could be uncovered, similar to decisions regarding food choice situations, where octopamine was shown to function in a switch-like manner (Claßen and Scholz, 2018).

Materials and Methods

Materials

Fly Stocks and Fly Husbandry

Table 1: List of fly lines used for the experiments.

Genotype	**Chromosome**	**Origin**
Larval Experiments		
CantonS		Lindsley and Zimm
w^{1118}	X	Lindsley and Zimm
Imago Experiments		
w^{1118}	X	Lindsley and Zimm
w^{1118},$T\beta h^{nM18}$/*FM7-GFP*	X	Henrike Scholz
*w**,$T\beta h^{nM18}$/*FM7;Tdc2-Gal4*	X;II	Manuela Ruppert
w^{1118},$T\beta h^{nM18}$/FM7-GFP; ;UAS-GlyS-RNAi	X;III	This study
w^{1118};*Tdc2-Gal4*	II	Jay Hirsh, BDSC 9313
w^{1118};*FB-Gal4*	II	Lee and Park, 2004, BDSC 33832
w^{1118};;*mef2-Gal4*	III	BDSC 27390
w^{1118};*FB-Gal4;mef2-Gal4*	II;III	This study
UAS-InR^{ca}	II	Exelixis, Inc. BDSC 8263
UAS-InR^{dn}	III	Exelixis, Inc. BDSC 8253
UAS-GlyS-RNAi	III	Perkins et al. 2015, BDSC 34930
UAS-GlyP-RNAi	III	Perkins et al. 2015, BDSC 33634

Animals were raised on ethanol-free standardised food containing thread agar, yeast, cornmeal, water, sugar beet molasses propionic acid and nipagin. Animals were raised

in plastic food vials at 25°C with a relative atmospheric humidity of approximately 60 % in a 12 h/12 h day-night cycle. All flies were backcrossed to w^{1118} (Scholz lab) for 5 generations.

Chemicals

Table 2: List of chemicals used during the experiments.

Chemical	**Company/Source**
Larval Experiments	
Agarose	VWR, A2114
D-Fructose	Sigma, F0127-1KG
n-amyl acetate (AM)	Sigma-Aldrich
Benzaldehyde (BA)	Fluka, 12010
2-heptanone (2-Hep)	Sigma-Aldrich, 02376-1ML
Ethyl acetate (EA)	Sigma-Aldrich, 58958-1ML
Acetic Acid (AA)	Sigma-Aldrich, 71251-1ML
1-Octanol (1-Oct)	Sigma-Aldrich, 95446-1ML
Paraffin oil	Sigma-Aldrich, 76235
Ethanol absolute (EtOH)	VWR, 20821.365
Periodic Acid Schiff's Staining Kit (PAS)	Sigma Aldrich, 1.01646.0001
Bovine Serum Albumin (BSA)	Sigma, A2153-50G
37 % Formaldehyde	Sigma, F8775-25ML
Imago Experiments	
D-Sucrose	Sigma-Aldrich, 84097
D-Arabinose	Acros Organics CAS: 28697-53-2
Yeast Extract	AppliChem A1552.0500
Strawberry red food colour	Ruth GmbH, E124
3-Octanol (3-Oct)	Sigma-Aldrich, 93856
4-Methyl-cyclohexanol (MCH)	Sigma-Aldirch, 153095
Paraffin oil	See above

Octopamine hydrochloride	Sigma, O0250-1G
Epinastine hydrochloride	Sigma, E5156-10MG
Glucose (HK) Assay Kit	Sigma-Aldrich #GAHK20-1KT
D-Amyloglycosidase	Sigma, A9913-10ML
NaCl	AppliChem GmbH

Solutions

Table 3: Solutions used for the experiments.

Solution	**Chemicals**	**Concentration**
Larval Experiments		
Phosphate Buffer Saline (pH 7.4) (PBS)	NaCl KCl Na_2PO_4 KH_2PO_4	137 mM 2.7 mM 10 mM 2 mM

The Tully and Quinn Olfactory Conditioning Apparatus

For olfactory associative learning one must combine an olfactory cue with an appetitive or aversive cue to analyse a behavioural shift of the animal towards the odorant. This can be achieved in *Drosophila melanogaster* with the use of an olfactory conditioning apparatus. The principle of the apparatus is based on the classical conditioning experiments performed by Ivan Pavlov (Pavlov, 1927) and the machines schematics are closely related to the machine described by Tully and Quinn in 1985. Hence, the machine is called the Tully and Quinn Olfactory Conditioning Apparatus, which will be abbreviated as the Tully machine from this point on.

Because this thesis focuses in large parts on the role of the internal state as a modulator of memory formation, the Tully machine was used to train *Drosophila* male flies to associate an odorant with a sucrose or yeast reward.

The Tully machine consists of 4 training positions, where custom-made training tubes can be applied that carry the animals within them. Below every training position a testing position can be found, where custom-made testing tubes can be applied. Flies can be transported through the machine via an elevator that can be put into different positions. A vacuum pump is necessary to produce a constant suction of approximately

2.5 lpm to provide a constant flow of odorant and air through the machine. The Tully machine should be positioned in a chamber that allows control of temperature and humidity. In a standardised setting, experiments are carried out at room temperature (approx. 23°C) and 80 % atmospheric humidity. This is especially important for aversive training, to intensify the electric shock.

2 M Sucrose is the most commonly used reinforcer, however many different concentrations and substances have been tested over the years (Huetteroth et al., 2015; Schwaerzel et al., 2003; Tully and Quinn, 1985; Tully et al., 1994; Wu et al., 2013). For aversive learning an electric shock of 90 V over 1 minute in 1.5 seconds of shock, followed by 3.5 seconds of rest pattern, repeated 12 times, is often used. For aversive learning there are also many different variations to the protocol (Iliadi et al., 2017; Pauls et al., 2010; Perisse et al., 2016; Tully and Quinn, 1985). An odorant, also called conditioned stimulus (CS), that is paired with the unconditioned stimulus (US, e.g., sugar or electric shock), is called the CS+. The odorant that is paired with the neutral stimulus is called the CS-. The basic principle of associative training is that training should start with the neutral or aversive stimulus followed by the positive stimulus. In appetitive training, flies encounter a dry filter paper paired with an odorant that can be applied from the outside to the training tubes. In aversive training, flies encounter the electric shock first. Afterwards the second odorant is paired with the appetitive reinforcer for appetitive learning and without electric shock for aversive learning. The exact protocols that were used in this work will be described below in another chapter.

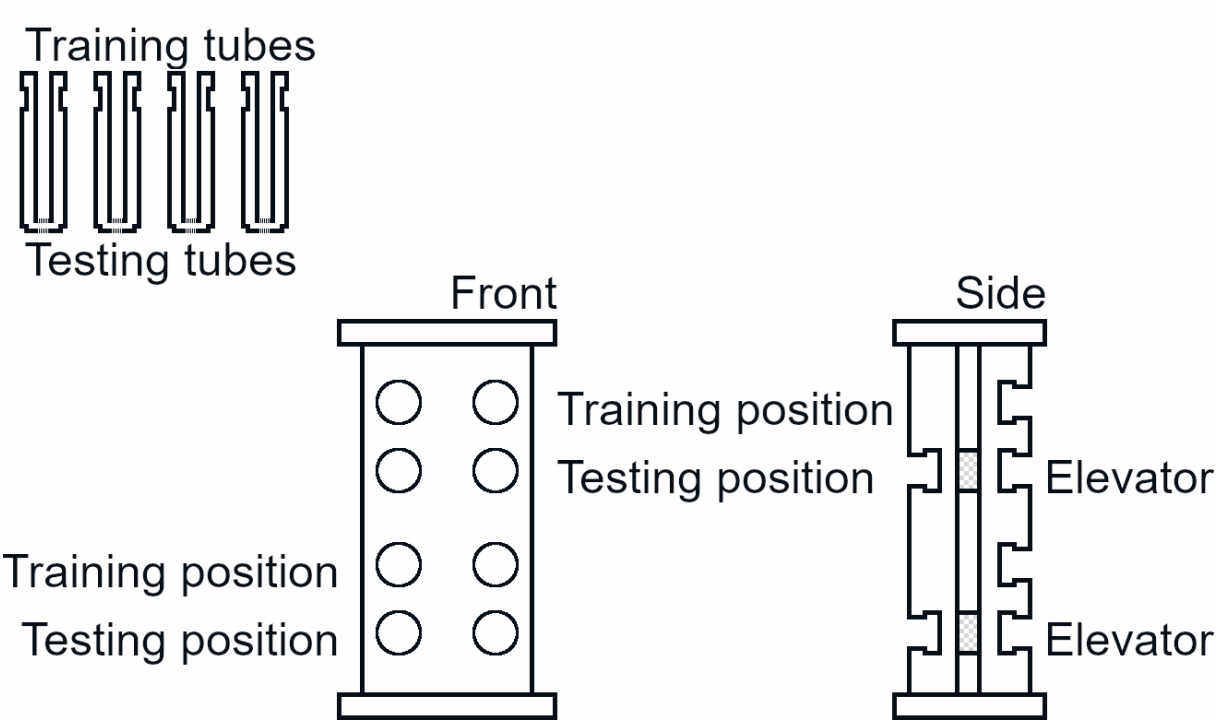

Figure 7: Tully and Quinn Olfactory Conditioning Apparatus Scheme

Schematics of the machine with training and testing positions for working with up to 4 groups simultaneously. In the side view the elevator ca be seen (grey). The middle part can be moved up and down and locked into place between the training and testing positions. This allows the experimenter to prepare the testing positions. The training position is only open in one direction, which forces the flies to encounter a stimulus and/or odorant without a choice. The testing position however allows a two-way choice for the animal. After training flies can walk to the front or the back, where they will encounter different odorants.

Larval behavioural assays

Crosses for behavioural experiments were set up in a density-controlled manner, with 15 male flies and 35 female virgin flies per big cultivation vial. Third-instar larvae were collected for behavioural experiments.

Olfactory preference and balance

Odorant preference was analysed, using a standardised behavioural paradigm (Schumann et al., 2021). Briefly, 20 larvae were collected with a brush out of a bit of food paste and collected in a drop of water to remove food paste residue.
Afterward, the larvae were transferred to petri dishes filled with 2.5 % agarose. Agarose was either plain, or contained varying concentrations of ethanol (5 %, 8 %, 10 %) or 2

M fructose. Ethanol containing agarose had to be hand warm before the ethanol was added and poured into the petri dishes.

In addition, the petri dish also carried two custom-made plastic odorant cups, one filled with an odorant, diluted in paraffin oil or pure, and the other one with plain paraffin oil. In the case of the odorant balance assay, both cups were filled with different odorants to assess whether both odorants were equally attractive or aversive to the larvae.

A perforated lid was placed on the petri dish and the larvae were transferred under the hood. A custom-made cardboard chimney was placed over the petri dish to deprive the larvae of potentially behaviour-altering light sources. Larvae had 5 minutes to freely explore the new surroundings and afterward, they were counted on either side of the petri dish. *Drosophila* larvae normally crawl towards odorants (Saumweber et al., 2011). Thus, a preference index (PI) could be calculated.

$$PI = \frac{\#Odorant\ side - \#Paraffin\ side}{\#Larvae\ (including\ neutral\ zone)}$$

A neutral zone of 2 cm, separating the petri dish in two halves, was defined. Larvae within those 2 cm were treated as undecided.

The same formula can be used to calculate larval odorant balance, to determine whether the odorants used in olfactory learning assays were of equal valence.

The odorants were diluted with paraffin oil in the following manner for larval olfactory behavioural assays, if not noted otherwise: AM (1:100), BA (undiluted), 2-Hep (1:300); 1-Oct (undiluted); EA (1:4000); AA (1:8000). Ethanol as the olfactory cue was analysed by mixing ethanol with paraffin oil. If applicable, diluted odorants were additionally supplemented with 5 % or 8 % ethanol.

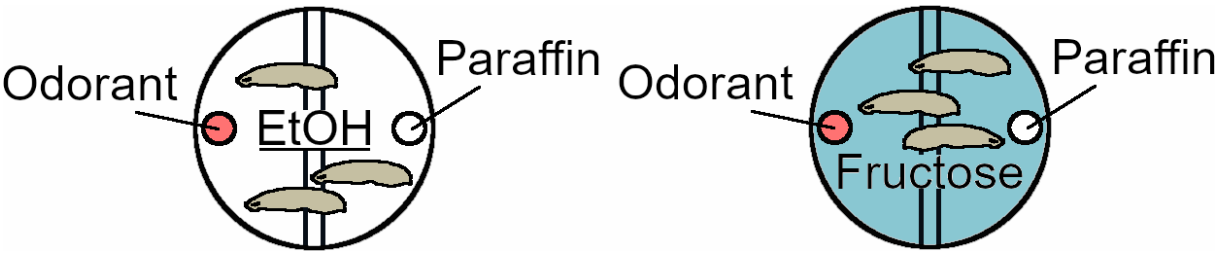

Figure 8: Olfactory preference scheme

20 larvae are put on a petri dish filled with agarose plus 0 %, 5 %, 8 % or 10 % ethanol or 2 M Fructose. For olfactory preference one of the odorant cups on the petri dish is filled with an odorant, the other one with plain paraffin oil. For odorant balance, both odorant cups are filled with an odorant to assess potential preferences between both odorants.

Larvae crawl freely on the petri dish for 5 minutes, in the dark and under the hood to reduce the effects of the outside world on behaviour. Afterwards the larvae are counted, and the PI is calculated. The two central lines on the petri dish in the figure symbolize the neutral zone.

Substrate preference

Larval preference or avoidance of ethanol containing crawling surfaces was assessed on petri dishes that were half filled with 5 % or 10 % ethanol-containing agarose and another half filled with plain agarose after the ethanol containing agarose had hardened. 20 larvae were placed on the petri dish, either on the ethanol containing side or on the plain side. A perforated lid was placed on top of the petri dish and the larvae were transferred under the hood and under the cardboard chimney. 5 minutes later the larvae were counted on either side of the plate to calculate a PI.

$$PI = \frac{\#Ethanol\ side - \#Plain\ side}{\#Larvae}$$

Differing from olfactory preference and balance assays, no neutral zone was determined prior to the experiments.

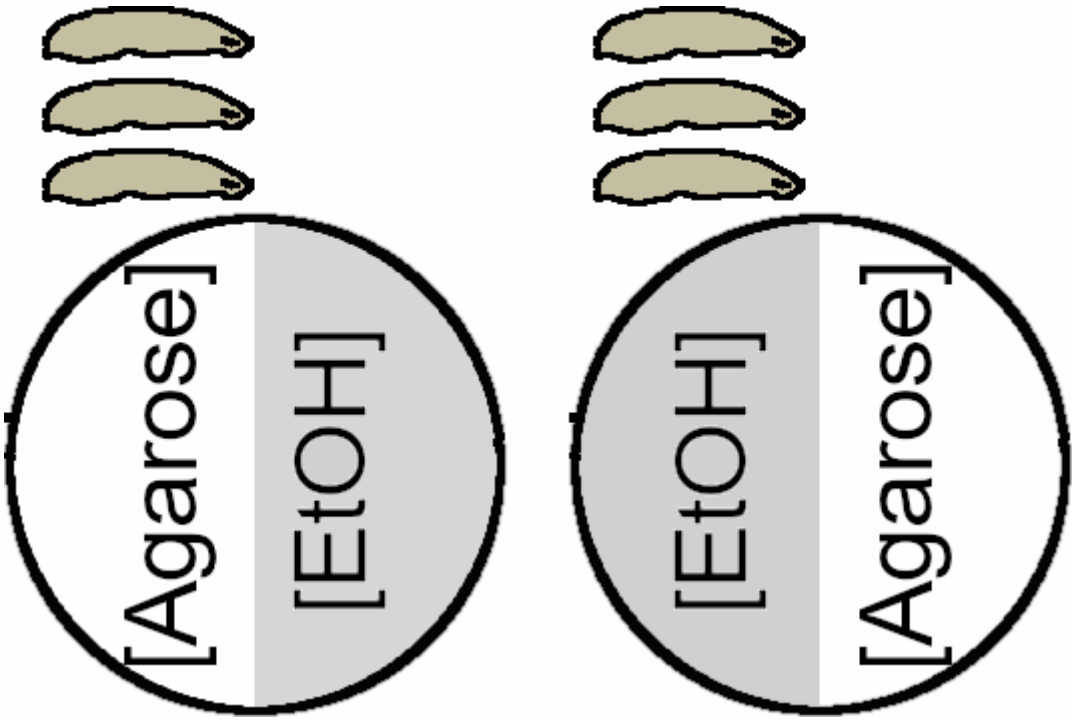

Figure 9: Substrate preference scheme.

20 larvae were collected and placed on petri dishes which were filled with agarose. One half was mixed with ethanol and let to dry, afterwards the second half was poured into the petri dish. The second half only consists of plain agarose. Larvae were either placed on the plain agarose side or the ethanol containing side and put under the hood in the dark for 5 minutes. Afterwards the larvae were counted on each side and the PI was calculated.

Odorant valance shift assay

The changes of valance of odorants after exposure were analysed with a modified olfactory one-cycle or three-cycle learning following previously published protocols (Weiglein et al., 2019; Widmann et al., 2016). Briefly, to start one trainings-cycle 20 larvae were collected, washed in a drop of water to remove food paste residue, and put on 2.5 % plain agarose plates. This agarose plate carried two custom-made odorant cups filled with either AM (1:100) or BA (undiluted). Larvae were transferred with the petri dish under the hood and under the cardboard chimney. A 5-minute timer was started. 1 minute before the timer ran out, the petri dish was taken from under the hood and the larvae were transferred on another plain agarose plate, carrying two custom-made odorant cups filled with the second odorant. Larvae were transferred under the hood again and the 5-minutes timer was started anew. A trainings-cycle consists of two petri dishes with different odorants which larvae must encounter for approximately 5 minutes.

For one-cycle training, the plate was taken from under the hood when 1 minute remained on the timer. The larvae were placed on a third plain agarose plate, carrying two custom-made odorant cups, one filled with AM (1:100) and the other one filled with BA (undiluted). The third plate is the testing plate. When all larvae were transferred on the fresh plate with both odorants, the petri dish was put under the hood again. 5 minutes later the larvae were counted to calculate a PI.

For three-cycle training, the plate was taken from under the hood when 1 minute remains on the timer. The larvae were transferred back to the first plain agarose plate and put under the hood again to start the second trainings cycle. Larvae must encounter the first and second plates three times before they were transferred to the third plain agarose plate. On the testing plate, both trained odorants are presented on the opposite side of the plate, equally to the test during one-cycle valance shift assay. 5 minutes later larvae were counted, and the PI was calculated.

$$PI = \frac{\#Odorant\ on\ 1st\ plate - \#Odorant\ on\ 2nd\ plate}{\#Larvae\ (including\ neutral\ zone)}$$

Training was performed reciprocally with two independent groups of 20 larvae simultaneously. One group started training with AM (1:100) and the other group started with BA (undiluted) on the first plate and was transferred to the second plate with the other odorant.

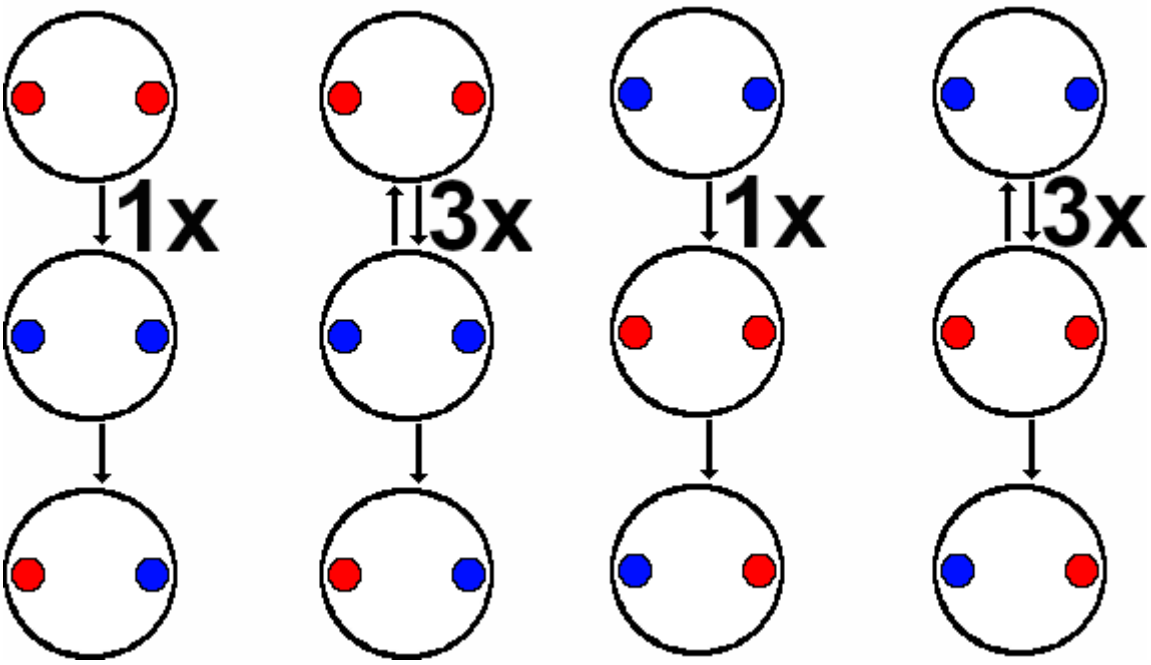

Figure 10: Odorant valance shift assay scheme

20 larvae were collected and placed in a petri dish filled with 2.5 % plain agarose. The plate carries two custom-made odorant cups, that are filled with an odorant. After 4 minutes of free crawling in the dark under the hood, when 1 minute remains on the timer, larvae are transferred onto a second plate with a second odorant and placed in the dark again. The timer is set for 5 minutes.

For one-cycle training, larvae are transferred, when the timer reaches 1 minute, onto a third plain agarose plate with both previously encountered odorants present and can choose for 5 minutes in the dark whether they prefer one odorant over the other.

For three-cycle training, larvae are transferred, when the timer reaches 1 minute, onto the first plate again and put back under the hood. This will be repeated until they have encountered each of the two odorants three times. Afterwards they are tested on a third plain agarose plate in the presence of both odorants to see whether the preference for the odorants had shifted. Larvae are counted on each side and the PI is calculated after the test.

Larval olfactory learning

To investigate the role of ethanol as a reinforcer for the *Drosophila* larvae, the larval olfactory learning assay was utilised (Schumann et al., 2021; Widmann et al., 2016). Generally, the assay is like the procedure described above (see Odorant valance shift assay), with the difference that one of the plates was supplemented with ethanol of varying concentration.

Briefly, 20 larvae were collected and put on an agarose plate that was either plain or paired with 5 %, 8% or 10 % ethanol and carried odorant cups that contained either AM (1:100) or BA (undiluted). Ethanol functions as the reinforcer in the training paradigms described in this chapter. A perforated lid was placed on top of the petri dish and the plate was transferred under the hood and under the chimney for 5 minutes. When 1 minute remained on the timer, the larvae were transferred to the second plate, carrying the second odorant. The second plate was, in relation to the first plate, either plain, or supplemented with 5 %, 8 % or 10 % ethanol. The larvae were put under the hood again and remained there for 5 minutes.

For one-cycle learning, larvae were transferred to the third plate, which was the testing plate. This plate was a plain agarose plate and carried both trained odorants. Larvae were transferred under the hood again. 5 minutes later the plate was taken out, the larvae on either side and within the neutral zone were counted and the PI was calculated.

For three-cycle learning, larvae were transferred back to the first plate to begin the next trainings cycle. After the third cycle was completed, larvae were transferred from the second plate to the third plate. Depending on the paradigm, the test was either performed directly afterwards or 5 minutes later. This testing plate could be plain agarose, or could be mixed with 5 %, 8 % or 10 % ethanol. The plate carried both trained odorants or a novel odorant substituting for one of the trained odorants. The testing plate was put under the hood and under the chimney and larvae had 5 minutes to freely crawl on the petri dish. 5 minutes later the plate was taken out, the larvae on either side and within the neutral zone were counted and the PI was calculated.

$$PI = \frac{\#AM - \#BA}{\#Larvae\ (including\ neutral\ zone)}$$

Figure 11 provides an overview of the training and testing plate combinations.

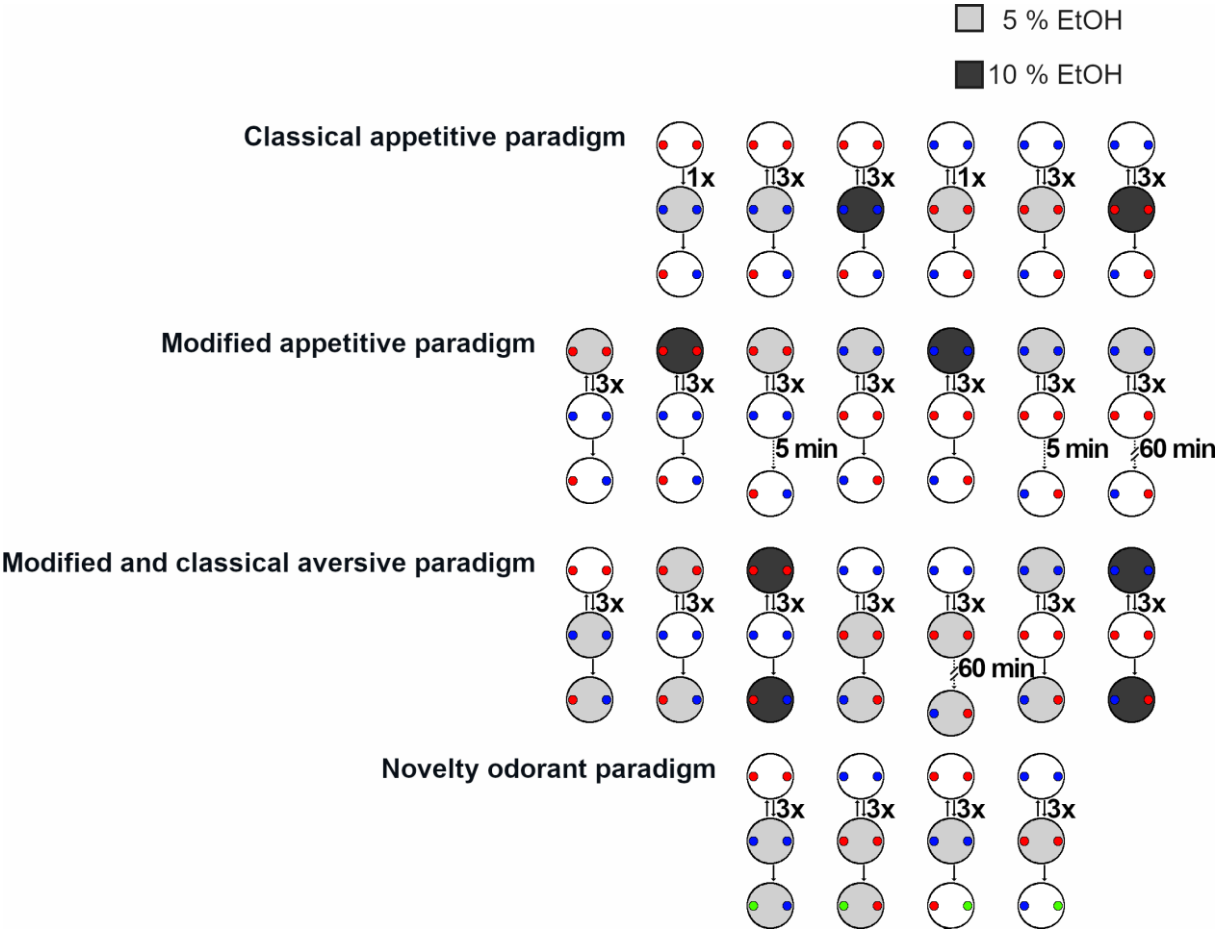

Figure 11: Larval olfactory learning schemes

Larval learning was performed as pictured in this figure. Larvae started either on plates carrying two odorant cups filled with AM (1:100) or BA (undiluted). After 5 minutes in the dark larvae were transferred on a second plate, carrying the other odorant, which was not trained in step 1. The training was either stopped after step 2 or repeated with step 1 for at least 3 times. Depending on the paradigm, either the first or the second trainings plate was supplemented with ethanol (Here only 5 % and 10 % are pictured). For an appetitive paradigm the testing plate was plain agarose. For an aversive paradigm, the testing plate contained ethanol.

The longer, dotted line in the modified appetitive paradigm describes a waiting phase of 5 or 60 min between training and test. For the novelty odorant paradigm, one of the trained odorants was exchanged for a novel odorant during the test.

Adult behavioural assays

3 – 5 days old, male flies, where not otherwise noted, were collected under CO_2 anaesthesia, and trained 2 days later, to remove the influence of CO_2 onto behaviour.

Different from larvae, adult *Drosophila melanogaster* flies are normally averse to high concentrations of certain odorants. Still, odorants can be used as conditioned stimuli (CS) in classical conditioning with fruit flies.
Olfactory learning behaviour and its associated control experiments can be analysed in the Tully machine (Tully and Quinn, 1985).

Odorant avoidance and balance

To perform odorant avoidance and balance experiments, approximately 70 male flies are collected per “n”. After 2 days without CO_2 anaesthesia flies are loaded into the trainings position of the Tully machine. The elevator is pulled up and the flies are transported into the elevator. While the flies are trapped in the elevator, the testing position is prepared with two testing tubes.
One odorant is applied to one end of the T-maze shaped testing position. On the other side a cup filled with paraffin oil is applied. The elevator is pulled down to the testing position, the bright light is turned off and a 2-minute timer starts. Flies can choose one of the branches of the T-maze freely. After 2 minutes the elevator is pulled up again, trapping flies in either one of the arms of the T-maze. The odorant cups are removed, and the flies are transferred into collection vials and are counted afterwards.
For odorant balance, the procedure is like the olfactory avoidance paradigm. However, flies choose between two sides, each carrying one of the two odorants that are used in olfactory conditioning assays. The odorants are diluted as follows: 3-Oct (1:80) and MCH (1:100) in paraffin oil.
The PI can be calculated.

$$PI = \frac{\#Odorant\ A - \#\ Paraffin/Odorant\ B}{\#Flies}$$

Appetitive Reinforcer Approach

For appetitive olfactory associative learning, flies are trained to pair an unrelated odorant with an appetitive reward, the unconditioned stimulus (US), that reinforces the behavioural shift towards the CS after training.
To test whether the chosen reinforcer is indeed attractive for the flies, the appetitive reinforcer approach assay must be performed.

For this 2 M Sucrose (approximately 69 %) or 5 % yeast extract are prepared the day before the experiment and 4 rectangular filter papers are soaked in the solutions. As an alternative offer for the choice situation, 4 rectangular filter papers are soaked in plain water. All filter papers are left to dry overnight.

16 h before the experiment the collected flies are transferred from food vials to starvation vials, where they are only offered water to prevent dehydration. Starvation is necessary to increase the attraction of the appetitive stimulus and to provide the proper motivation to perform the task (Tempel et al., 1983).

The next day the filter papers are rolled up and put into custom-made testing tubes. 70 male flies are loaded into the trainings position with a tube that is unrelated to either of the trainings tubes that will be used during the choice situation. Flies are trapped in the elevator and the testing position is prepared by adding the sucrose or yeast filter paper carrying trainings tube on one side and the dried water filter paper carrying trainings tube on the other side. The bright light is turned off, the elevator is pulled down and the flies are released into the choice situation.

Here they now have 2 minutes to choose one side over the other. After 2 minutes the elevator is removed, thus leaving the flies trapped on either side of the elevator. The training tubes are removed, and the flies are transferred to collection vials and counted after the end of the experimental set up. The calculation of the PI follows.

$$PI = \frac{\#Sucrose/Yeast - \#Water}{\#Flies}$$

Appetitive olfactory short-term memory training paradigm

Approximately 70 male flies were collected 2 days prior to the experiment and kept in medium sized food vials. 16 h or 40 h prior to the start of the training session, flies were transferred to medium size empty food vials with a Whatman paper soaked with 200 µl of water (water was replenished after 24 h for 40 h starved flies). 4 filter papers were prepared and soaked with 0.15 M Sucrose, 2 M Sucrose or 5 % yeast solution. 4 additional filter papers were prepared by soaking them with water. All filter papers were dried overnight.

The vacuum pump and climate chamber were turned on as described in the chapter “Tully and Quinn Olfactory Conditioning Apparatus” prior to the experiment, filter papers were put into the training tubes and flies were arranged next to the workspace

to ensure smooth and easy handling during the training sessions. Odorants were diluted as previously described. The odorants were 3-Oct (1:80) and MCH (1:100).
Flies were loaded into the first training tubes, which carry the blank filter papers and put into the 4 corresponding training positions in the Tully machine. The bright light was exchanged for red light, to reduce visual cues for the animal. A timer was started which ran for 1 minute to give the animals time to adjust to the new situation. After 1 minute the odorant cups were applied, carrying one of the two training odorants. This odorant is now the CS-. A timer was started for 2 minutes.
When the timer reached zero, the odorant cups were removed (beginning with the cup that was put in position first). The first set of training tubes was removed with the flies in them. The flies were transferred from the blank filter paper training tubes to the sucrose filter paper training tubes and the training tubes were put back into the corresponding training positions.
20 - 30 seconds of adjustment was given to the animals before the second set of odorant cups, with the second training odorant, were applied. This odorant is now the CS+. A timer was started for 2 minutes.
After the 2 minutes, the odorant cups were removed, beginning with the cup that was first put in position. For short-term memory analysis, the elevator was pulled up and the flies were transferred to the elevator. For this the Tully machine must be put at an angle of approximately 90° to induce upwards crawling of flies. When the animals fall into the elevator, the elevator can be moved down into a position between training and testing.
The Tully machine is put back into position and the testing situation can be prepared by adding the 8 testing tubes (4 at the front, 4 at the back) and applying the two trained odorants. 4 odorant cups to the front, 4 odorant cups to the back. The elevator is brought down, the animals are released into the choice situation, the red light was turned off and a 2-minute timer was started. The test was performed in complete darkness to further reduce potential visual stimuli. After the 2 minutes, the elevator was pulled up, flies were trapped on either side of the choice situation and the bright light was turned on again. The odorant cups were removed, and flies were put into collection vials and counted after the training and testing cycles were complete.

For long-term memory analysis however, flies were put back into the starvation vials before the testing phase. After the first exposure to the second training odorant, flies are removed from the Tully machine for 6 hours.

To test the animals 6 hours later, flies were loaded into the training position with fresh training tubes to reduce potential bias towards the type of tube. The elevator was pulled up, flies were loaded into the elevator and the testing position was prepared by adding the testing tubes and the odorants. Flies were released into the choice situation by pulling the elevator down and turning off the red light. A timer was started for 2 minutes. After the 2 minutes, flies were trapped on either side of the elevator by pulling the elevator up, the odorant cups were removed, and the flies were collected into collection vials for counting.

The Learning index (LI) was calculated as follows:

$$LI = \frac{(\#CS+) - (\#CS-)}{\#Flies}$$

To each group of 70 flies a reciprocal group of naïve flies was trained and tested as well to calculate an odorant independent LI by calculating the mean between both LI. When for one group 3-Oct (1:80) was the CS+, the reciprocal group is trained with MCH (1:100) as the CS+.

Figure 12 gives an overview of the training and testing steps.

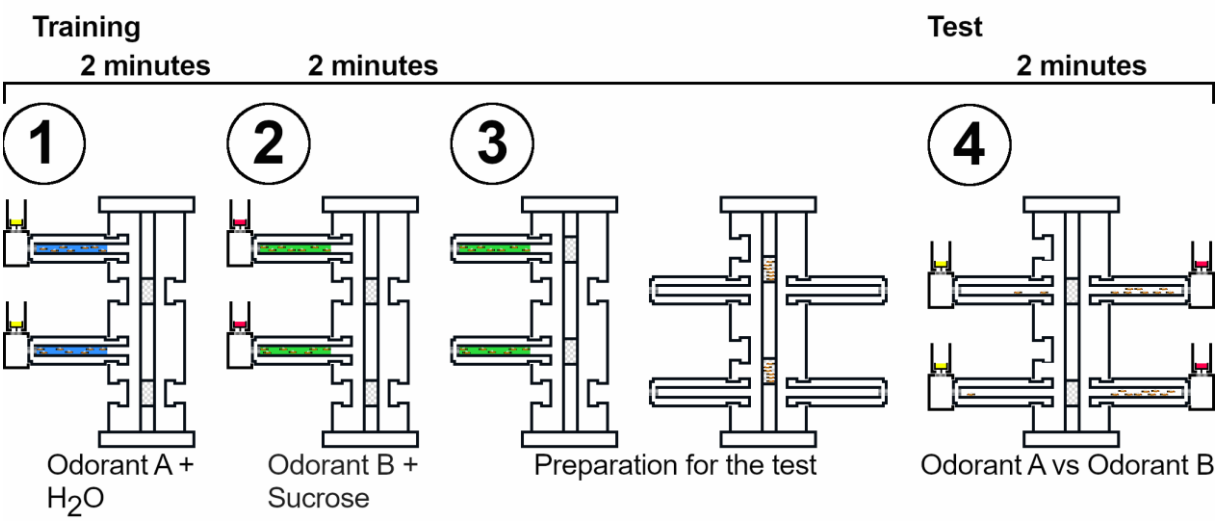

Figure 12: Appetitive short-term memory training paradigm scheme

70 flies were loaded into each of the four training positions of the Tully machine (1). The first odorant, CS-, was applied. 2 minutes later the odorant cups were removed, and the flies were transferred into a second set of training tubes, with sucrose or yeast-soaked filter papers in them. A second odorant, the CS+, was applied (2). After 2 minutes, flies were either put aside for 6 hours to test for LTM, or the elevator was pulled up and the flies were transferred into the elevator to give time to prepare the testing positions (3). The test was performed by applying the CS- odorant on one side and the CS+ odorant on the other side of the Tully machine. Flies got 2 minutes to choose between the two odorant (4). Afterwards the elevator was removed, the flies were trapped and collected into collection vials for further counting.

Cold-shock anaesthesia resistant memory and long-term memory assays

Approximately 70 male flies were collected 2 days prior to the training. To reduce the potential effects of CO_2 anaesthesia, flies were kept at 25°C for 2 days. 16 h or 40 h before the experiment began, flies were transferred to empty medium sized food vials. A Whatman paper at the bottom was supplemented with 200 µl H_2O and provided moisture. For 40 h starved flies, water had to be replenished after 24 h.
One day before the training, 4 filter paper were prepared by soaking them in 2 M Sucrose or water and drying them overnight.
Flies were trained in a similar manner to the short-term memory training paradigm in the previous chapter. Briefly, flies were transferred into training tubes that carried the dried blank filter paper, 1 minute after the transfer the odorant cups were applied. After 2 minutes, the odorant cups were removed again; flies were transferred into the second set of training tubes and were put back into the training positions.
The second set of odorant cups was applied 20 – 30 seconds later. Flies got 2 minutes with the second odorant. Afterwards the odorants were removed.
To distinguish between protein synthesis independent anaesthesia resistant memory (ARM) and de novo protein synthesis dependent long-term memory (LTM) flies can be cold-shocked at different time points between training and testing (Eschment et al., 2020; Krashes and Waddell, 2008).

To analyse ARM, flies were removed from the Tully machine after training and were put back into the starvation vials for 2 hours. Afterwards flies are transferred into glass vials, which were put on ice. Flies underwent cold-shock treatment for 2 minutes and were put back into starvation vials afterwards. Shortly after the cold-shock, flies recovered. They spent another hour on starvation before they were tested.

To analyse LTM, flies were removed from the Tully machine and put directly into the cold-shock treatment. 2 minutes later, they were put back into starvation vials for 3 hours. To test both sets of flies, they were transferred into the training positions. The elevator was pulled up and flies were trapped in it between training and testing positions. The testing position was prepared by applying the testing tubes and the CS+ on one side and the CS- on the other side.
Flies were released into the choice situation and the red light was turned off. A 2-minute timer was started. Afterwards, flies were trapped on either side of the elevator by pulling the elevator up. The odorant cups were removed, and flies were transferred into collection vials for counting.
The LI was calculated. To each group of 70 flies a reciprocal group of naïve flies must be tested as well to calculate an odorant independent LI by calculating the mean between both LI. When for one group 3-Oct (1:80) was the CS+, the reciprocal group was trained with MCH (1:100) as the CS+.

Weight measurements

A 2.5 ml Eppendorf tube was weighed prior to the weight measurements and the scales were tared afterwards to remove the weight of the Eppendorf tube from the gained numbers. 100 male flies of every genotype were collected, anaesthetised with CO_2, and put in a 2.5 ml Eppendorf tube for measurements. The weight measurements were repeated at least 5 times with 100 independent male flies of each genotype. When necessary, flies were starved 16 or 40 h prior to weight measurements.

Capillary feeder assay

To analyse the consumption of nutrients in adult *Drosophila melanogaster* males, the Capillary Feeder (CAFE) assay was utilised (Ja et al., 2007). Experiments were conducted with 3 – 5 days old flies.

3h CAFE assay

The 3 h CAFE assay measures the consumption rate of adult *Drosophila* male flies regarding their hunger. 20 male flies were collected for each “n” and starved 16 or 40 h prior to the beginning of the experiment. During starvation and the experimental

procedure, flies were kept in medium sized vials with a wet filter paper (approximately 200 µl of water were added to prevent desiccation). Before the experiment was started, the number of surviving animals at the beginning of the experiment was counted. Per vial 4 capillaries were filled with nutritious solutions of different composition. Either a solution was prepared with consisted of 5 % sucrose, 2 % red food dye (1:10 diluted) and water, or 5 % yeast extract, 2 % red food dye (1:10 diluted) and water, or 5 % sucrose, 5 % yeast extract, 2 % red food dye (1:10 diluted) and water.

Before the consumption assay started, each capillary was marked at the meniscus. This way the consumed amount of liquid can be measured. In addition to the vials carrying male flies, 3 empty vials also carrying capillaries filled with solution were added as evaporation controls. In addition, 4 vials were filled with water and put in the corners of the CAFE box. A lid was put on top of the CAFE box and the box was placed at 25°C with 60 % atmospheric humidity for 3 h.

Afterwards the box was removed from the 25°C room and the capillaries were pulled individually, and the new meniscus was marked. The consumed amount of solution was calculated with Microsoft Excel for Microsoft 365, Version 2202. Flies were removed and the materials from the capillary feeder assay were washed and dried for further use.

24 h CAFE assay

The 24 h CAFE assay measures the consumption rate of adult *Drosophila* male flies regarding their appetite. 8 3 – 5 days old male flies were collected for each “n” of the 24 h CAFE assay.

The procedure was like for the 3 h CAFE assay, with the exception that animals normally did not need to be starved. However, to analyse animal behaviour and changes in appetite when adult flies were pre-starved, flies were starved 16 h and 40 h for 24 h CAFE as well. Animals were put in medium sized CAFE vials either shortly before the experiment began (when not starved) or 1 – 2 days before the experiment were performed. When animals were starved for a long time, water had to be replenished after approximately 24 h. Before the experiment started, the number of surviving animals at the beginning of the experiment was counted. The rest was prepared, performed, measured, and calculated like in the 3 h CAFE assay, only 24 h after the beginning of the experiment and not 3 h.

CAFE assay calculations

The amount of consumed solution was measured in mm with an electronic calliper. Each measured value was divided by 14.6. This number was given by the manufacturer to convert mm in µl of consumed solution. For every CAFE box the evaporation controls were independent of other CAFE assays performed on the same day. A mean was calculated from the evaporation controls and subtracted from each µl value of the consumed solution.
The total consumption per vial was calculated by adding up all four capillaries. Next, the total consumption of every fly was calculated by dividing the total consumption per vial by the number of flies. Last, the total consumption per µg fly was calculated by dividing the total consumption per fly by the mean weight of a fly of the corresponding genotype and starvation time.

Histochemical and molecular biological Assays

Periodic Acid Schiff Reaction

To visualise stored carbohydrates in the organs of *Drosophila melanogaster*, larvae were used for Periodic Acid Schiff Reaction. First, L3 larvae were dissected in 1 % ice cold Bovine Serum Albumin (BSA) in 1x Phosphate Buffer Saline (PBS). Larvae were removed from the food paste, washed with water, and put on ice for a short period to anaesthetise the animals. Minutia needles were placed right below the mouth hook of the larvae and at the bottom to immobilise them. Dissection scissors were used to make a vertical cut from one needle to the next and the resulting muscle filets were pinned down with additional needles.
The fat body was removed and stored in 1 % BSA in 1x PBS solution, while the other organs were removed completely to get a clean body wall muscle preparation. Muscle tissue was fixed with 3.7% Formaldehyde in 1 % BSA in 1x PBS for 20 minutes on the dissection dish, while the fat body was fixed in a 1.5 ml Eppendorf tube. The Eppendorf tube was put on a rocking platform to be kept in motion.
After the fixation the tissue was washed 2x with 1 % BSA in 1x PBS for 5 minutes. The minutia needles were removed, and the filet was transferred into a 1.5 ml Eppendorf tube. At that point Eppendorf tubes carrying the fixed tissue were put under a small cardboard box to prevent light interfering with the experiment during incubation and

kept in motion on a rocking platform. Periodic acid solution was added to the Eppendorf tubes for 5 minutes. Next, the tissue was washed 2x for 5 minutes with 1% BSA in 1x PBS. The washing solution was removed, and Schiff's reagent was added for 5 minutes.
Afterwards, the tissue was washed again 2x for 5 minutes in 1 % BSA in 1x PBS and mounted in 50 % Glycerol in 1x PBS.
Pictures were taken with AXIO Scope.A1 from Zeiss and the Axio Vision Rel. 4.8 from Zeiss.

Glycogen Measurement

To analyse whole-body glycogen levels in adult *Drosophila* the commercially available Glucose (HK) Assay Kit was used, following a protocol by Tennessen et al. published in 2014. Briefly, 5 male flies were collected in 1.5 ml Eppendorf tubes, and washed with ice-cold PBS to remove food paste residues, when applicable. 100 µl ice-cold PBS was added to the Eppendorf tube and the flies were thoroughly homogenised with the motor homogenisator (Roth: 9748.1). Next, the samples were put into an incubator for 10 minutes at 70° C. Afterwards, the samples were centrifuged at 4° C, at maximal speed for 3 minutes. The supernatant was transferred into fresh 1.5 ml Eppendorf tubes.
Next, amyloglucosidase stock solution (AS) was prepared, by adding 1.5 µl amyloglucosidase to 1 ml 1x PBS. Additionally, the glucose standard must be diluted, by adding 16 µl glucose standard to 84 µl 1x PBS. Subsequent dilutions of 0.16 mg/ml, 0.08 mg/ml, 0.04 mg/ml, 0.02 mg/ml, and 0.01 mg/ml are necessary for the linear standard curve.
Next, fly samples must be diluted 1:3, by adding up 20 µl of sample and 40 µl of 1x PBS. Two sets of Eppendorf tubes are needed next, one with 20 µl 1x PBS, and the other one with 20 µl AS.
20 µl of 1:3 diluted sample were added to both sets of Eppendorf tubes, this way having a total dilution of 1:6.
Next, 30 µl of 1x PBS were added as blanks, to a 96-well plate. Additionally, 30 µl of 1:6 diluted PBS and AS treated samples and the glucose standard curve dilutions were added.

The 96-well plate was incubated at 37° C for 60 minutes. Afterwards, 100 µl of HK reagent was added to the wells and incubated at room temperature for 15 minutes. Levels of total glucose were photometrically measured at 340 nm with the ELISA reader. Glycogen levels were calculated by subtracting the total glucose of 1x PBS treated samples from total glucose of AS treated samples.

Statistical analysis

Learning behaviour and all related control data was presented as box plots with the middle line symbolising the median, the lower border of the box was the 25 % quartile, and the upper border was the 75 % quartile. The whisker represents 1.5x of the interquartile range (IQR). Food consumption behaviour was presented as bar plots, representing the mean of each data set. The error bars were the ± s.e.m. Difference from random choice was calculated with parametric one-sample t-test. Difference between two groups was calculated with the parametric student's t-test. Difference between three or more groups was calculated with the parametric one-way ANOVA with post-hoc Bonferroni Holm Correction. Statistical analyses were performed with Statistica 9.1 (StatSoft, Tulsa, OK, USA) as well as some homepages (listed below). Boxplots were created with Excel 2016 and visually modified with GIMP 2.10.12.

One-Way ANOVA with post-hoc Bonferroni Holm Correction: https://astatsa.com/OneWay_Anova_with_TukeyHSD/

One-sample t-test:
https://www.graphpad.com/quickcalcs/oneSampleT1/

Results

The role of ethanol as reinforcer in olfactory conditioning in *Drosophila melanogaster* larvae

During development, *Drosophila melanogaster* larvae encounter ethanol in their natural habitat. They hatch and live on an ever-increasing amount of ethanol until they pupate and emerge as an imago. Ethanol provides nutrition, protection against parasites and can also be used as a teaching signal and is therefore thought to be attractive to the animal (Geer et al., 1985, 1989; Kacsoh et al., 2013; Milan et al., 2012; Schumann et al., 2021). However, a systematic analysis of the role of ethanol as an olfactory associative conditioning reinforcer was never performed. A study from last year appears to be the first approach into a systematic analysis of ethanol and larval relationship (Schumann et al., 2021), showing an appetitive reinforcer role for ethanol. Therefore, this thesis provides a first systematic approach to analyse the significance of ethanol as an environmental cue and reinforcer in *Drosophila melanogaster* larvae.

CantonS and w^{1118} are attracted to different ethanol concentrations

To analyse the relationship of larvae towards ethanol, a general attraction to ethanol concentrations had to be established. Thus, olfactory attraction assays were performed, with different ethanol concentrations presented on one side of a plain agarose plate and undiluted paraffin oil presented on the other side (Figure 13).
CantonS larvae show indifference towards 5% EtOH and 10% EtOH (Figure 13A). However, they significantly prefer 8% EtOH. w^{1118} show a clear and significant preference for 5% EtOH, which they do not have for 8% EtOH (Figure 13B). Here they show indifference. In contrast to *CantonS*, 10% EtOH was significantly avoided.
Taken together, the results show that *Drosophila* larvae show a genotype specific attraction towards specific ethanol concentration and that the *white* mutation in w^{1118} shifts the attraction towards lower ethanol concentrations.

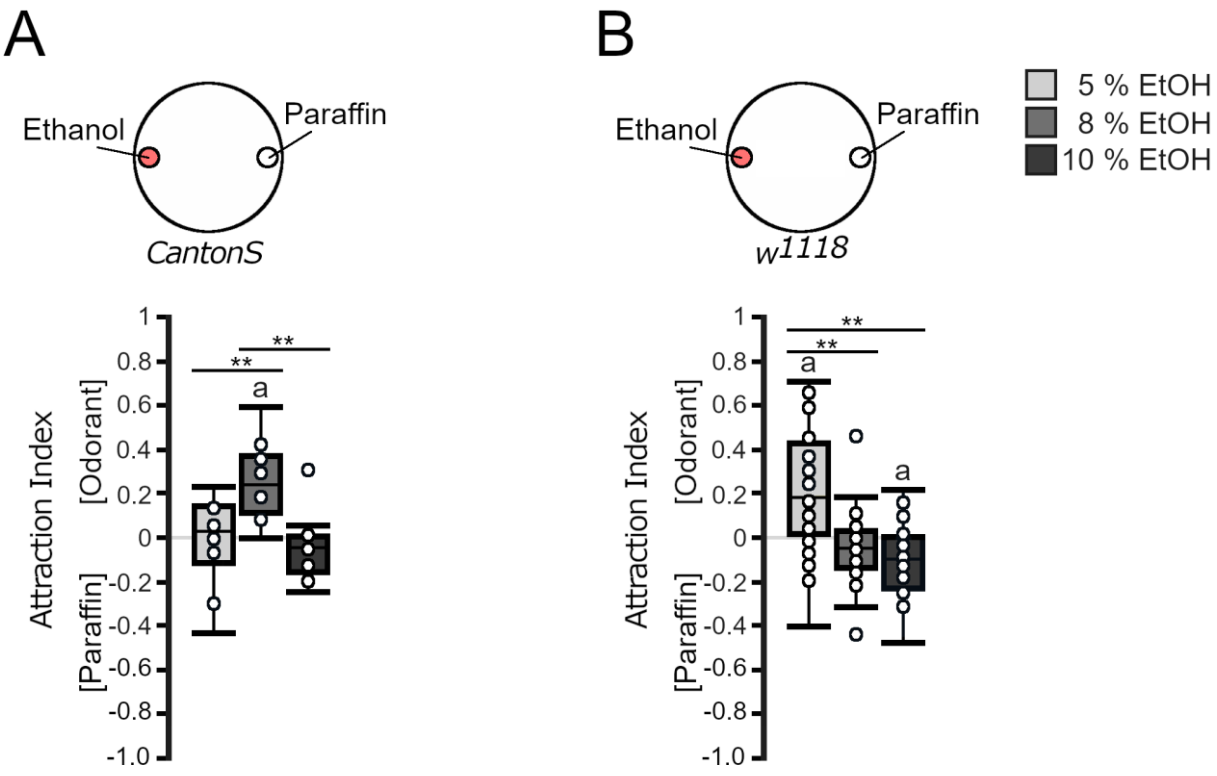

Figure 13: Larvae are attracted to ethanol as an odour source in a concentration dependent manner.

Larvae started in the centre of the plate. **A**, *CantonS* larvae show indifference to 5% EtOH, significant attraction to 8% EtOH and indifference to 10% EtOH. N = 10,10,12. **B**, w^{1118} larvae show significant attraction to 5% EtOH, are indifferent to 8% EtOH and show significant aversion to 10% EtOH. N = 20, 20, 20.

Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student’s t-test. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Ethanol influences innate odorant attraction in *CantonS* but not w^{1118} larvae

Normally, larvae are attracted to nearly all sources of olfaction (Aceves-Piña and Quinn, 1979) especially key-odorants that carry innate information about potential food sources (Giang et al., 2017). To investigate the effects of ethanol on larval olfactory behaviour, it was of interest to see how larvae would react to attractive odorants in the presence of ethanol. Therefore, standardised olfactory attraction assays on agarose plates with different ethanol concentrations mixed into the agarose were performed (Figure 14).

Based on the results seen in Figure 13, 0%, 5% and 8% EtOH for *CantonS* larvae and 0%, 5% and 10% EtOH for w^{1118} larvae was used.

All groups show significant attraction towards the used odorants independent of genotype or ethanol concentration in the agarose plate.

CantonS show significant attraction to 1-OCT (pure) on agarose plates and on ethanol containing agarose plate. Interestingly, attraction towards 1-Oct (pure) is not altered by EtOH presence in the agarose. *CantonS* larvae show a significant increase in attraction towards AM (1:100) on 5% EtOH in comparison to plain agarose (Figure 14A). However, on 8% EtOH plates, the attraction towards AM (1:100) is significantly decreased in comparison to 0% and 5% EtOH plates. Attraction towards BA (pure) is significantly reduced on 8% plates. Although, there is no significant difference for BA (pure) preference between 0% and 8% EtOH. The attraction towards 2-Hep (1:300) was also significantly decreased on 8% EtOH. Interestingly, although larvae of w^{1118} prefer lower ethanol concentrations (Figure 13), the presence of ethanol in the crawling surface did not affect their attraction towards all used odorants (Figure 14B).

Thus, w^{1118} appears to be a better suited candidate than *CantonS* to analyse ethanol guided olfactory association behaviour, because the reinforcer did not influence the perception and preference for the conditioned stimulus in these experiments.

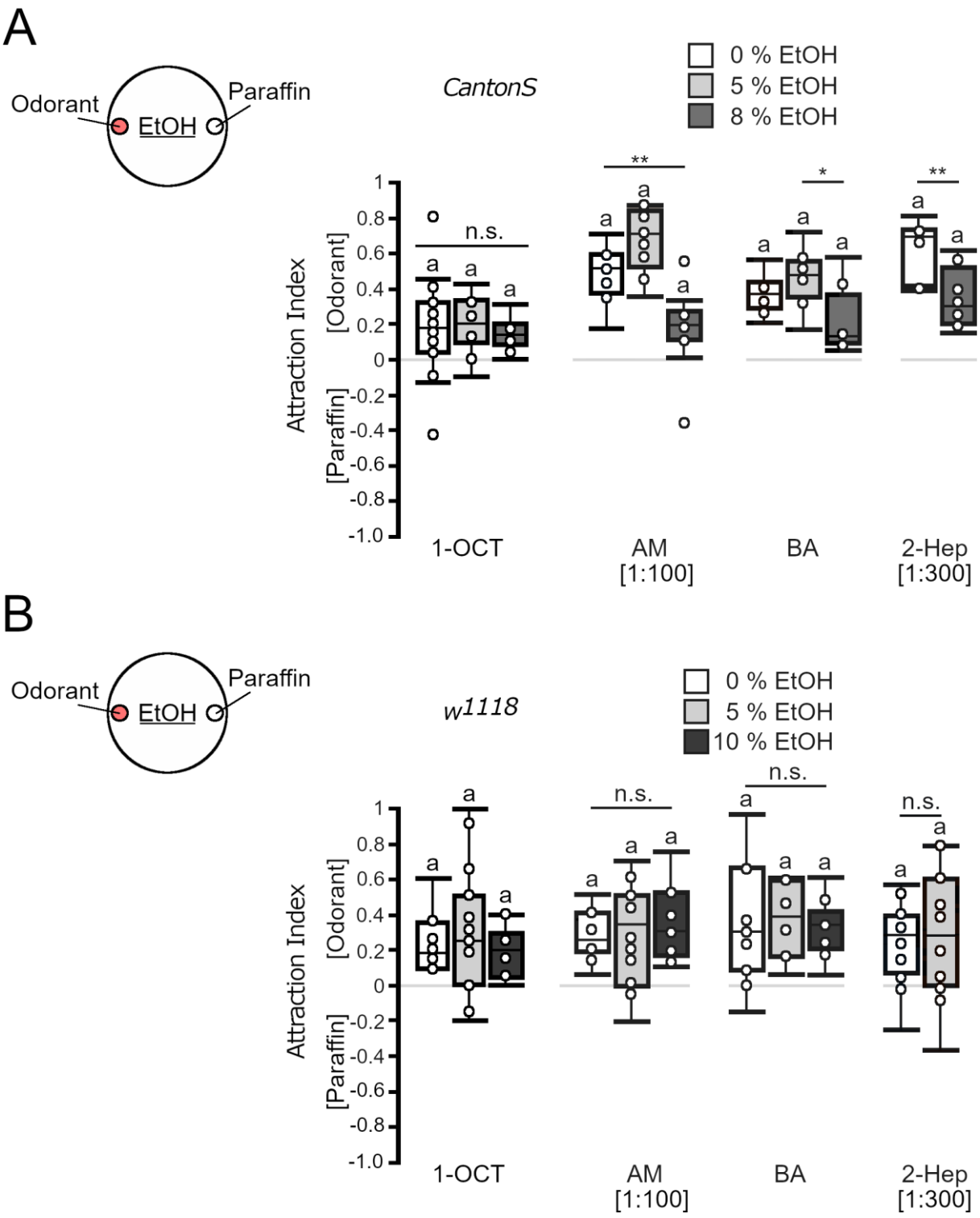

Figure 14: Ethanol presence influences odorant attraction in *CantonS* larvae

A, an odorant was presented to on one side of an agarose plate mixed with 0, 5 or 8% EtOH for 5 minutes. On the other side plain paraffin oil was presented at the same time. *CantonS* larvae show a significant preference for all presented odorants. However, they show a decrease in odorant attraction in the presence of 8% EtOH for all cases, except 1-Oct. N = 19, 10, 10; 8, 9, 12; 8, 8, 8; 8, 8, 8. **B**, an odorant was presented to on one side of an agarose plate mixed with 0, 5 or 10% EtOH for 5 minutes. On the other side plain paraffin oil was presented at the same time. Larvae of w^{1118} show no significant

changes in their attraction to odorants, with or without the presence of EtOH in the crawling surface. N = 10, 19, 10; 10, 14, 10; 11, 8, 9; 12,12.
Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student’s t-test. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Low doses of ethanol in the crawling surface are attractive to the larvae

Higher concentrations of ethanol significantly altered odorant attraction in *CantonS* but not w^{1118} larvae. Additionally, ethanol itself appears to be an attractive smell in a concentration dependent manner for *CantonS* but not for w^{1118}. Therefore, it was interesting to investigate the side preference of *CantonS* and w^{11118} larvae, on low (5%) and high (10%) concentrations of ethanol to also investigate whether crawled on ethanol is attractive.
Thus, side preference assays were performed, where groups of larvae could freely choose between a plain agarose side and an ethanol containing side (Figure 15).
When *CantonS* larvae start their exploration on the plain agarose side, they tend to go towards the middle of the plate at the edge between the agarose and ethanol containing site (Figure 15A). Interestingly, *CantonS* did not crawl towards the middle line, when they started exploration on the ethanol containing side of the plate. This suggests that the presence of ethanol, independent of concentration, is preferred by the larvae more than being on plain agarose.
Larvae of w^{1118} show a similar behavioural pattern to *CantonS* when they start exploration on the plain agarose side. On 5% and 10% ethanol, larvae crawl towards the middle line, where a low dose gradient of ethanol can be expected (Figure 15B). They also remain on the ethanol side when they start exploring on 5% EtOH. However, in contrast to *CantonS,* w^{1118} show a clear and significant avoidance of the 10% EtOH side, when they start exploration on it. This is consistent with the overall attraction to lower doses of ethanol in w^{1118} larvae.
Taken together, these results suggest a general attraction towards low doses of ethanol contained in the crawling substrate.

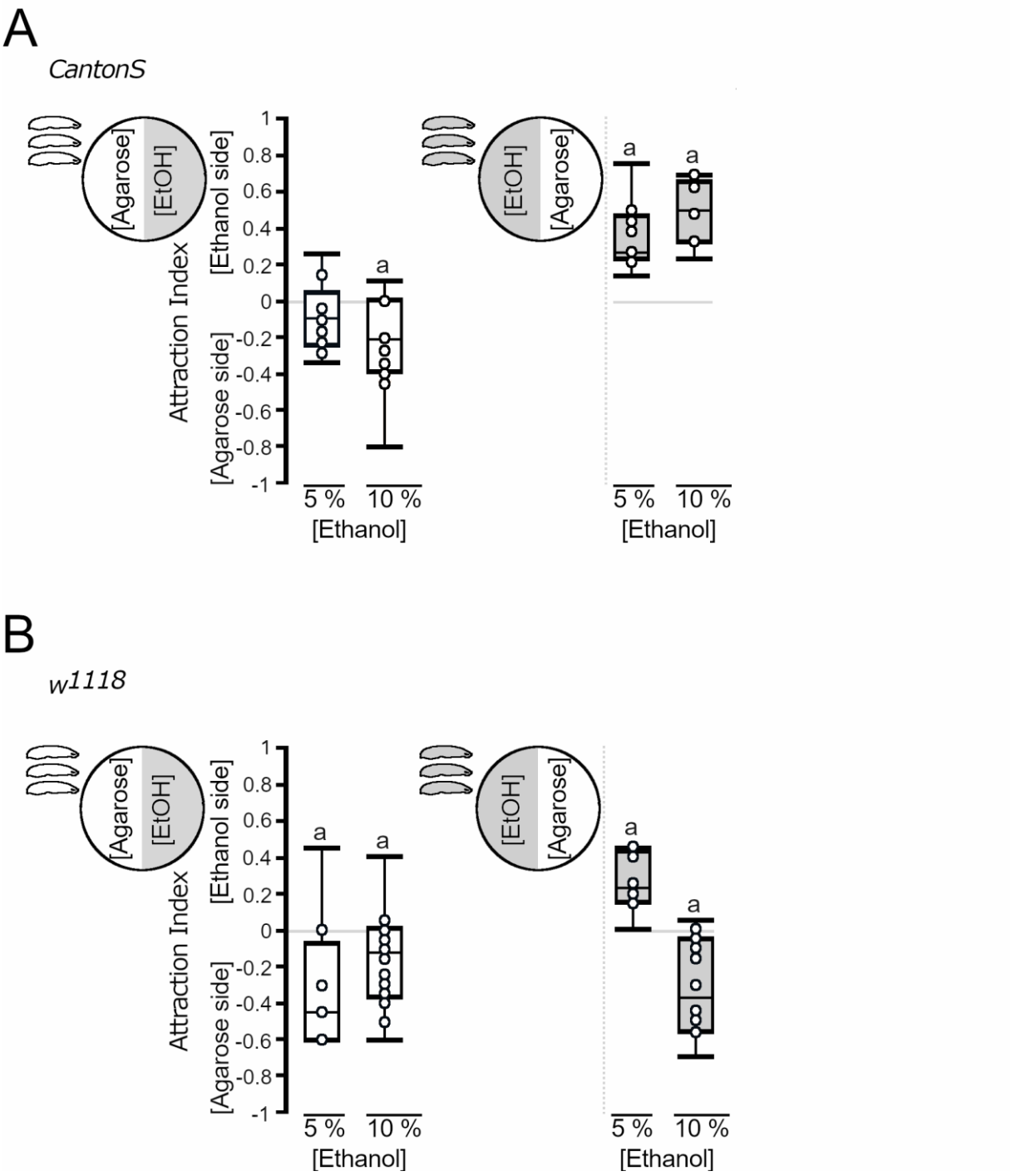

Figure 15: Low doses of ethanol in the crawling surface are preferred by the larvae

A, *CantonS* larvae started exploration either on the plain agarose or ethanol containing agarose (5% or 10%) and had 5 minutes to choose a side. Starting exploration on plain agarose, larvae show movement toward the middle of the plate, while, when they started exploration on the ethanol side, they remained on ethanol. N = 9,14; 9,9. **B**, w^{1118} larvae move to the middle line, when they start exploration on the plain agarose side. However, they remain on the 5% EtOH side but avoid the 10% EtOH side, when they start exploration on ethanol. N = 8,14; 8,14. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

Carbohydrate presence counteracts the attractive effects of ethanol and fermentation related odorants

Drosophila larvae show a preference for low-dose ethanol containing agarose and concentration dependent attraction to ethanol as an olfactory cue. Ethanol as a carbohydrate source is utilized by larvae to generate fatty acids (Freriksen et al., 1991; Geer et al., 1985). Larvae could be attracted to ethanol containing surfaces and the smell of ethanol because of the nutritional value of alcohol. Thus, it was interesting to investigate, whether an alternative carbohydrate source -e.g., fructose- could counteract the attraction of ethanol as an odorant (Figure 16).

To analyse larval behaviour towards ethanol on fructose containing surfaces, odorant attraction assays were performed, where larvae could choose between an odorant and paraffin oil on fructose plates and plain plates. The odorants used were, ethanol, AM (1:200), 2-Hep (1:300), AA (1:8000) and EA (1:4000). AA and EA were used as controls because AA is a key-odorant for fermentation processes and EA a key-odorant for yeast in general (Becher et al., 2012; Giang et al., 2017; Piškur et al., 2006).

w^{1118} larvae show a significant preference for 2-Hep and AM, odorants that are not related to ethanol and fermentation processes but are still present in nature (Hallem et al., 2004; Oppliger et al., 2000; Zhu et al., 2003) (Figure 16A). As expected, preference for 5% EtOH presented on plain agarose plates completely disappears in the presence of 2 M fructose in the agarose. Interestingly, while AA preference also disappeared on 2 M fructose, EA preference remained unaffected. This suggests that ethanol might function as an olfactory cue for fermentation processes, while yeast volatiles might code for another nutritional source, namely proteins.

To further investigate the link between ethanol and fructose as exchangeable carbohydrate sources, three-cycle trainings were performed with fructose reinforcement (Figure 16B, 16C). For this, additional odorant balance experiments were performed prior to the training to show that 5% EtOH and AM are equally attractive and that none of the odorants is preferred more, to ensure that both stimuli are perceived equally (Table 4). Although, AM (1:200) was statistically more attractive -as determined by the one-sample t-test- than 5% EtOH on 2 M fructose, this concentration was still used for the three-cycle training, because the odorants were at least balanced on the pure plate, while for every other concentration of AM a significant preference was always observed. However, this must be taken into consideration, when interpreting the associative olfactory conditioning.

Table 4: Sensory acuity tests for *w*^{1118} ethanol and fructose conditioning experiments

					Odorant Balance
	AM 1:100 v 5% EtOH	**AM 1:200 v 5% EtOH**	**AM 1:200 v 5% EtOH**	**AM 1:300 v 5% EtOH**	**AM 1:300 v 5% EtOH**
Genotype					
	Pure	**Pure**	**2 M fructose**	**Pure**	**2 M fructose**
w^{1118}	**-0.45 ± 0.09**	**-0.18 ± 0.09**	**-0.31 ± 0.12**	**-0.27 ± 0.06**	**-0.36 ± 0.11**
	******	**n.s.**	*****	******	*****
N	**8**	**9**	**10**	**10**	**9**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p <0.05; ** = p < 0.01.

Next, the olfactory conditioning was performed.

Larvae were trained in a three-cycle trainings paradigm starting on a 2 M fructose plate with either AM (1:200) or 5% EtOH as CS, following by a second step on a 2 M fructose plate with the other odorant, that was not used in step one (Figure 16B). This trainings-cycle was repeated 3 times. Both reciprocal groups show a significant attraction towards AM (1:200) after a three-cycle training when both odorants were offered on a plain agarose plate. Ethanol, paired with fructose, did not elicit attraction This suggests that either the larvae only formed a positive association with AM but not with EtOH, or that ethanol as an olfactory cue for carbohydrates was meaningless in the presence of a carbohydrate reinforcer during training.

To investigate the latter possibility, ethanol had to be distinguished from fructose. Larvae were thus trained reciprocally in a three-cycle trainings paradigm, starting on 2 M fructose plates and either AM (1:100) or BA (pure), an odorant pair that is commonly used in literature and can be sensed by the larvae (Huser et al., 2017; Kreher et al., 2005; Widmann et al., 2016). In the second step larvae were transferred onto 5% EtOH containing plates and presented with the second odorant. This trainings-cycle was repeated 3 times. Tests were performed on pure agarose plates.

Prior to the conditioning experiments, odorant acuity experiments were performed to verify that none of the odorants were more attractive than the other (Table 5). Indeed, neither AM (1:100) nor BA (pure) were significantly more attractive than the other odorant on pure plates, 2 M fructose plates and 5% EtOH plates. Thus, the odorant

pair were equally attractive or not attractive and suitable to be used for conditioning experiments.

Table 5: Sensory acuity tests for *w*^{1118} ethanol and fructose conditioning experiments

			Odorant Balance
	AM 1:100 v BA	**AM 1:100 v BA**	**AM 1:100 v BA**
Genotype			
	Pure	**2 M fructose**	**5 % EtOH**
w^{1118}	**-0.05 ± 0.11**	**0.11 ± 0.09**	**0.09 ± 0.07**
	n.s.	**n.s.**	**n.s.**
N	**9**	**10**	**8**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p <0.05; ** = p < 0.01.

Both reciprocal groups show a significant attraction to the odorant paired with 2 M fructose after a three-cycle trainings paradigm (Figure 16C). Therefore, it can be concluded that fructose is more attractive than ethanol after three-cycle, although the caloric value of both reinforcers is nearly equal, as determined following the values given by the FAO in 1998 (FAO, 1998; Pohl et al., 2012).

Taken together, ethanol and acetic acid attraction is suppressed in the presence of 2 M fructose, which suggests that this might be due to both odorants being signifiers for carbohydrate presence. Furthermore, ethanol was always not preferred after conditioning with fructose, either because the larvae were indifferent to it in the presence of fructose, or because other pharmacological effects of ethanol, like intoxication or cell toxicity might be aversive to *w*^{1118} larvae.

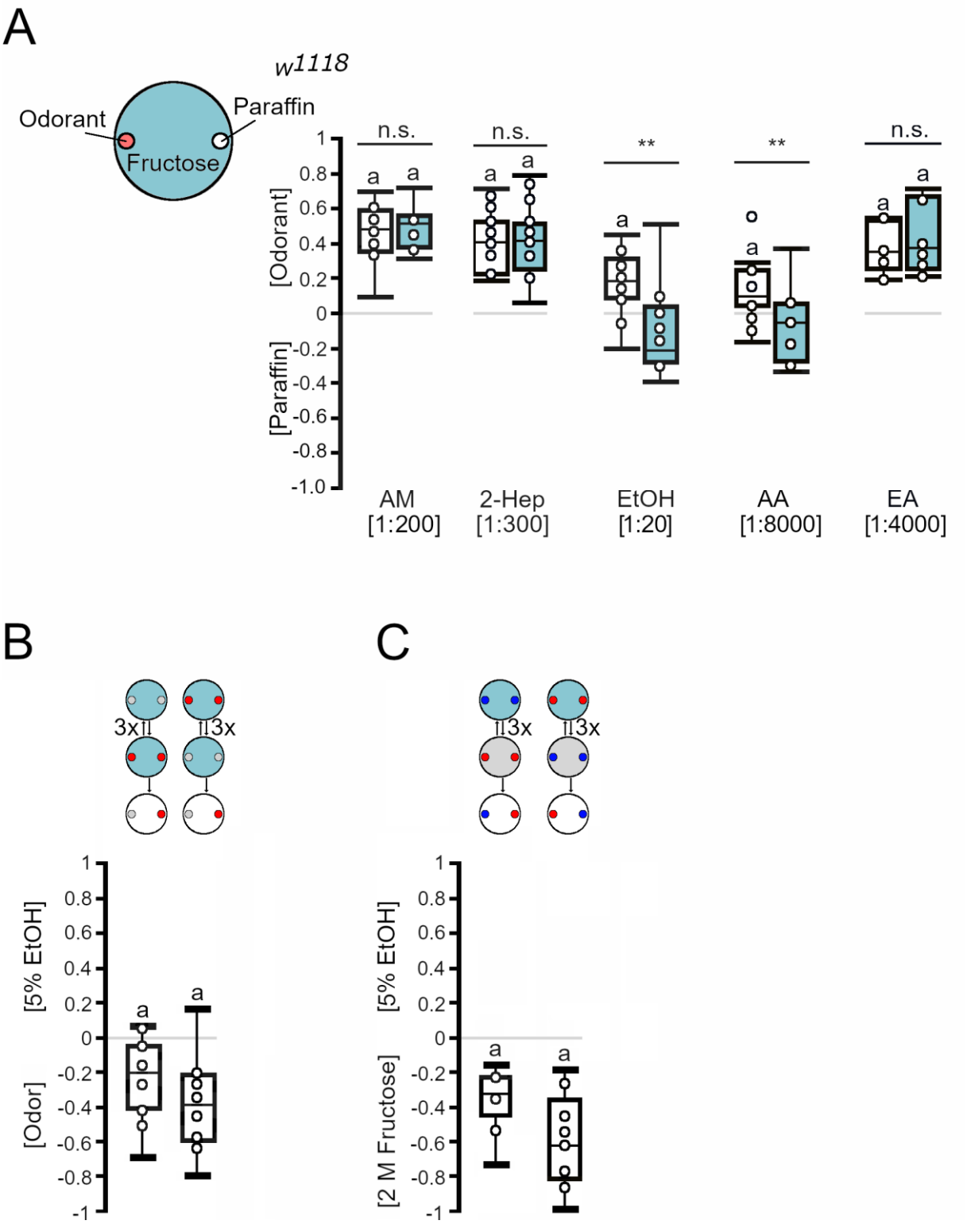

Figure 16: Ethanol and acetic acid attraction is suppressed in the presence of fructose.

A, larvae had 5 minutes to choose between an odorant (indicated in the figure) or paraffin oil on either plain agarose plates or 2 M fructose plates. Non-key odorant attraction to 2-Hep (1:300) and AM (1:200) was not suppressed by 2 M fructose presence, while 5% ethanol and key-odorant AA (1:8000) attraction was suppressed by fructose. However, attraction towards key-odorant EA (1:4000) was not significantly altered on 2 M fructose in comparison to plain agarose plates. N = 8, 8; 17, 15; 12, 12; 15, 15; 9, 9.

B, larvae were trained in a three-cycle trainings paradigm (reinforcer and odorants indicated in the figure) and tested on a pure agarose plate. Both reciprocal groups show attraction towards AM (1:200) after three-cycle training. N = 12, 12. **C**, larvae were trained in a three-cycle trainings paradigm (reinforcer and odorants indicated in the figure) and tested on a pure agarose plate. Both reciprocal groups show significant attraction to the odorant paired with 2 M fructose over three cycles. N = 9, 9. Difference from random choice was calculated with the one sample t-test. "a" above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student's t-test. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Odorant Balance is not affected by ethanol mixed into the odour cups

Ethanol presence in the agarose influences olfactory attraction in *CantonS* but not in w^{1118} third instar larvae. In odorant acuity experiments with both genotypes, larvae show no significant preference for AM (1:100) or BA, when they are left to explore for 5 min on an agarose plate (Figure 17B and 17C). The same applies for experiments where the odorant balance was analysed on different ethanol concentrations (5% and 10%) (Table 6). However, *CantonS* show significant preference for AM (1:100) on 5% EtOH, when the balance test is performed with AM (1:100) and 1-Oct. Furthermore, w^{1118} also show a significant preference for AM (1:100) on 5% and 10% EtOH plates, when the balance test is performed with AM (1:100) and 1-Oct. Because 1-Oct is also an alcohol, the behavioural conditioning experiments were performed with the pairing of AM (1:100) and BA.

Table 6: Odorant Balance experiments with *CantonS* and w^{1118}

			Odorant Balance	
	AM (1:100) v BA (pure)	**AM (1:100) v BA (pure)**	**AM (1:100) v 1-Oct (pure)**	**AM (1:100) v 1-Oct (pure)**
Genotype				
	5% EtOH	**10% EtOH**	**5% EtOH**	**10% EtOH**
CantonS	**0.18 ± 0.13**	**-0.05 ± 0.11**	**0.22 ± 0.08**	**0.006 ± 0.05**
	n.s.	**n.s.**	*****	**n.s.**
w^{1118}	**0.09 ± 0.07**	**0.19 ± 0.07**	**0.16 ± 0.05**	**0.22 ± 0.09**
	n.s.	**n.s.**	******	*****
N (*CantonS*/w^{1118})	**10/8**	**14/11**	**8/10**	**15/10**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p <0.05; ** = p < 0.01.

To determine, whether the influence of ethanol on odorant attraction and balance is limited to ethanol presentation in the agarose, balance tests were performed with ethanol mixed to the odorants into the cup (Figure 17). The ethanol concentrations that elicited significant attraction as an odorant in Figure 13 were used for the analysed genotypes. 8% for *CantonS* and 5% ethanol for w^{1118}. Results are presented as dot plots with each dot representing a group of 20 individuals.

While *CantonS* did not show a significant preference for either AM (1:100) or BA on agarose plates, they did significantly avoid BA, when it was mixed with 8% EtOH (Figure 17B), an ethanol concentration that they normally approach when they smell it (Figure 13). Although, mixing AM (1:100) with 8% EtOH and balancing it against BA did not lead to significant avoidance of AM (1:100), *CantonS* larvae still show a tendency to crawl away from the ethanol paired odorant.

To further investigate, whether *CantonS* larvae could discriminate between an odorant and the same odorant paired with ethanol, balance tests were performed where animals could choose between an odorant and the same odorant paired with 8% EtOH (Figure 17B). When BA is mixed with 8% EtOH, larvae significantly avoid that side of the agarose plate, while they show a balanced behaviour in case of AM (1:100) mixed with 8% EtOH against AM (1:100). These results show that BA (pure) paired with 8% EtOH is aversive, while they do prefer AM (1:100) with and without the ethanol.

Next, w^{1118} was also tested for their odorant balance behaviour with 5% EtOH mixed into different CS (Figure 17C). Fitting with the results of Figure 13, where w^{1118} did not show significant changes in odorant approach while on ethanol crawling surfaces, ethanol that was mixed into the odorant cups did not alter odorant balance between AM (1:100) and BA.

Taken together, *CantonS* appears to be more sensitive to behavioural adaptation induced by attractive concentrations of ethanol. Whether w^{1118} would also show behavioural changes at 8 % ethanol concentration was not analysed. This could be interesting to investigate further in the future.

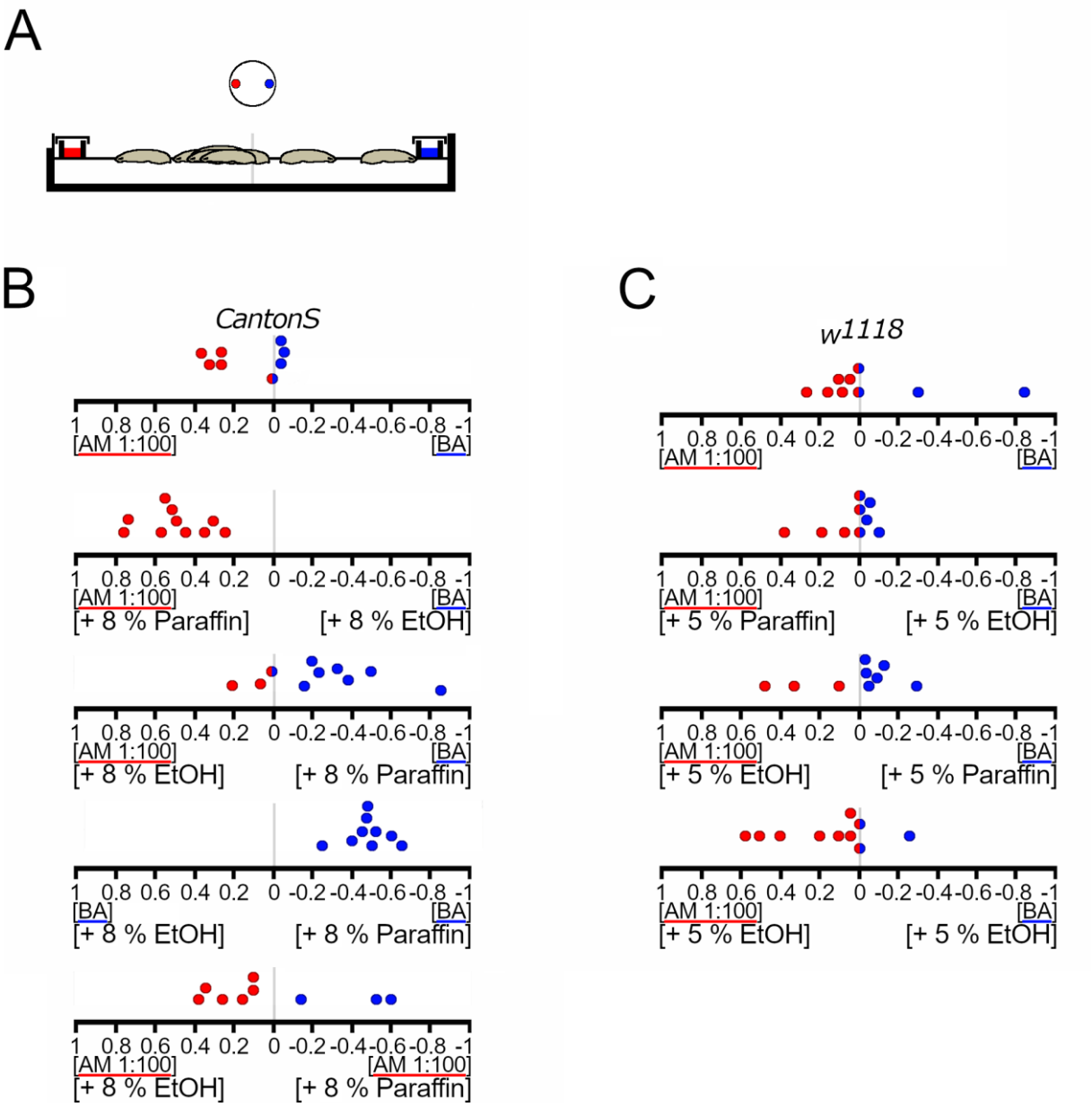

Figure 17: *CantonS* shows an ethanol presence related shift in odorant balance behaviour

A, schematic representation of a larval odorant balance experiment. **B**, odorant balance behaviour represented as dot plots for *CantonS* with different CS supplemented with 8% EtOH. BA (pure) mixed with ethanol leads to significant aversion of BA (pure), while AM (1:100) mixed with 8% EtOH was still equally attractive or aversive as BA (pure). N = 8, 10, 10, 9, 9. **C**, odorant balance behaviour represented as dot plots for w^{1118} with different CS supplemented with 5% EtOH. In all tested cases, AM (1:100) and BA (pure) were equally attractive 5 minutes after the beginning of larval exploration. N = 9, 9, 9, 10. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

Odorant attraction to AM (1:100) is reduced by 5% ethanol during conditioning

To investigate the role of ethanol as a reinforcer for olfactory conditioning for *Drosophila* larvae, olfactory conditioning experiments were performed following a standardised, appetitive trainings paradigm (Figure 18).

Larvae of w^{1118} show a shift in olfactory preference when they are exposed to AM (1:100) and BA in a one-cycle or three-cycle reinforcer-less trainings paradigm (Figure 18A). During the test, they show a significant and robust preference for AM (1:100). This preference is not altered, when BA is paired with 5% EtOH during training in a one-cycle or three-cycle trainings paradigm. A 5-minute delay before the test to interfere with the interstimulus-interval that also plays an important role in larval olfactory conditioning (Weiglein et al., 2021; Yarali et al., 2009) did not alter the exhibited preference for AM (1:100) after training. Thus, 5% EtOH paired with BA does neither reduce nor enhance attraction towards AM (1:100), which is also present without the reinforcer.

The reciprocally trained groups did start on BA and AM (1:100) and were paired in a second step with 5% EtOH (Figure 18B). It was not surprising to see significant attraction towards AM (1:100) after one-cycle and three-cycle training in a reinforcer-less conditioning paradigm. However, in a one-cycle training with AM (1:100) paired with 5% EtOH in the second step, w^{1118} larvae failed to show significant preference for AM (1:100) during test. Significant preference for AM (1:100) reinstates in a three-cycle training and in a three-cycle training with 5-minute wait before testing, although the overall scores were lower in comparison to the results in Figure 18A.

Therefore, ethanol appears to negatively affect attraction to AM (1:100) after conditioning. Taken together, ethanol paired with BA appears to be aversive to the animal, while ethanol paired with AM (1:100) reduced naturally occurring attraction to AM (1:100) after pre-exposure, implying that ethanol might be a negative reinforcer for w^{1118} larvae.

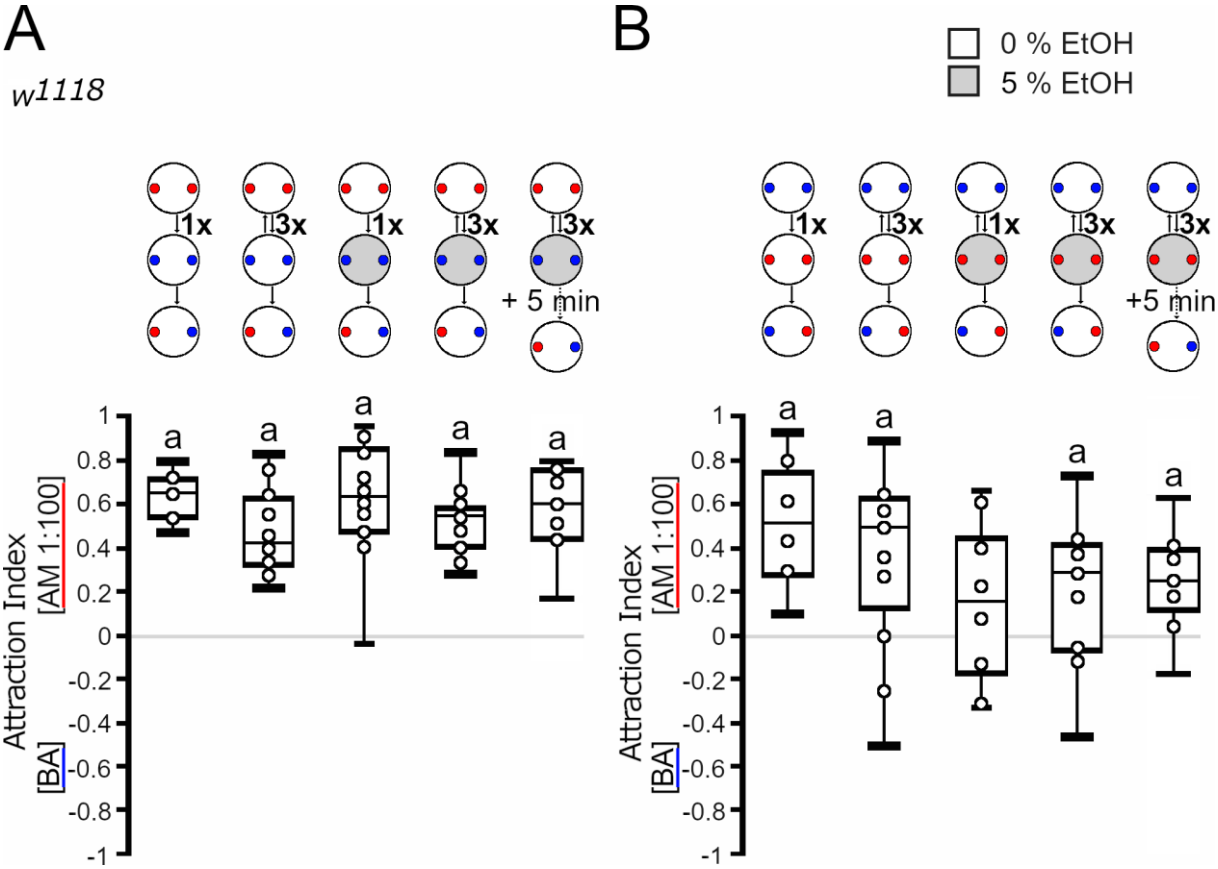

Figure 18: AM (1:100) attraction after pre-exposure is reduced when paired with 5% EtOH during training.

A, different conditioning paradigms without and with 5% EtOH reinforcement in the second step, paired with BA (pure). *w*^{11118} shows a preference for AM (1:100) after one- or three-cycle pre-exposure, which is not altered by pairing BA (pure) with 5% EtOH during conditioning. N = 8, 13, 10, 11, 9. **B**, different conditioning paradigms without and with 5% EtOH reinforcement in the second step, paired with AM (1:100). w^{1118} shows a preference for AM (1:100) after one- or three-cycle pre-exposure which is altered in a one-cycle training where AM (1:100) in paired with 5% EtOH in the second step. Significant attraction reinstates in three-cycle conditioning. N = 8, 13, 10, 11, 9. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

Odorant attraction to AM (1:100) persists for at least 60 minutes

Ethanol reinforcement during appetitive conditioning resulted in opposite behaviour regarding the CS+. To unravel, whether this effect is due to odorant-reinforcer interaction or because of the order in which the reinforcer is presented during training, larval olfactory modified appetitive conditioning experiments were performed, where the 5% EtOH reinforcer was presented in the first step and the tests were performed on agarose plates (Figure 19).

Figure 19A shows that the time-point during training when ethanol is presented in the agarose matters. When compared to figure 18, larvae w^{1118} show no decrease in attraction towards AM (1:100) when the CS+ is introduced in the first step of every cycle.

To investigate, whether this observed attraction towards AM (1:100) persists and might potentially be memory formed by three-cycle training, the time between training and testing was extended by 5 as well as 60 minutes. Memory, formed through a three-cycle training, should be stable enough to sustain that retention interval (Huser et al., 2017; Widmann et al., 2016).

Attraction towards AM (1:100) indeed persisted for at least 60 minutes and balance did not reinstate. Whether this is due to reinforcing properties of AM remains unclear.

Next, AM (1:100) was paired with 5% EtOH in the first step of conditioning. Here, the expected attraction towards AM (1:100) after training was observed in all groups (Figure 19B). Interestingly, the order of reinforcer presentation during appetitive conditioning, influences the strength of behavioural output during test, seeing that starting with AM (1:100) plus 5% EtOH reinforcement led to a decreased variance in the box plots in comparison to results that where produced, whenn the CS+ was presented in step 2 of conditioning (Figure 18B).

Taken together, the test-training interval influences behavioural outcome with ethanol reinforcement in an odorant specific manner that appears to be not a result of larvae forming memory with alcohol reinforcement.

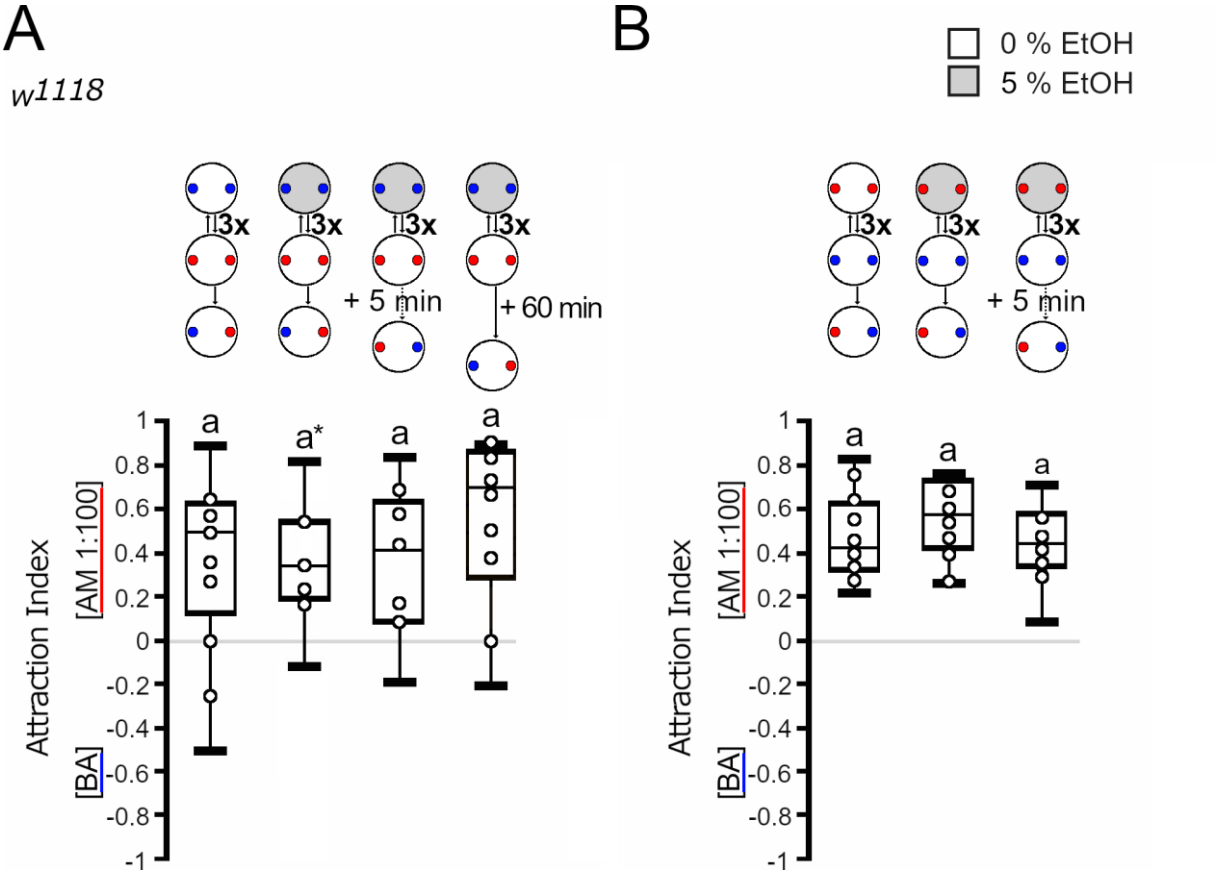

Figure 19: Ethanol reinforcement does not affect attraction to AM (1:100) in the modified appetitive conditioning assay

A, three-cycle modified appetitive olfactory conditioning with w^{1118} and 5% EtOH reinforcement. Figure 19A shows also results already seen in Figure 18 (most left box plot, reinforcerless conditioning) as a help to visualise the effects observed with this form of conditioning. Ethanol presented in the first position of a three-cycle training did not alter attraction towards AM (1:100). The attraction to AM persisted for 5 as well as 60 minutes after training. N = 13, 9, 9, 10. Boxplot marked with a star (19A second from the left) was produced with data produced and provided by Bariş Yapici. **B**, three-cycle modified appetitive olfactory conditioning with w^{1118} and 5% EtOH reinforcement. Figure 19B shows also results already seen in Figure 18 (most left box plot, reinforcerless conditioning) as a help to visualise the effects observed with this form of conditioning. AM (1:100) was approached after training, with and without 5% EtOH reinforcement. N = 13, 12, 9. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

Ethanol presence during test affects behavioural outcome in a paradigm specific manner

Drosophila larvae shift their behaviour during test after ethanol conditioning depending on the specific odorants paired with 5% EtOH reinforcement, which appears to be unrelated to appetitive memory formation normally occurring after similar training sessions with other reinforcers.

Thus, the question arose whether ethanol might function as an aversive reinforcer in classical and modified aversive conditioning (Figure 20).

When 5% EtOH reinforcement was paired with AM (1:100) in the second step of conditioning and the test was carried out directly or 5 minutes after training on reinforcer containing plates, larvae show behaviour that is not significantly different from random choice. Therefore, ethanol presence during the test interferes with AM (1:100) attraction after repeated exposure. However, 60 minutes waiting between conditioning and test reinstates significant AM (1:100) attraction after pre-exposure. Furthermore, classical aversive conditioning resulted in attraction towards AM (1:100), although it was paired with 5% EtOH in the first step. This shows that the order in which AM (1:100) as CS+ is presented during conditioning, influences behavioural outcome. To verify learning and thus the reinforcing capabilities of ethanol, BA (pure) should produce similar results after modified and classical aversive conditioning. Contrary to the expectations, BA (pure) as CS+ was always aversive to the larvae after conditioning. Thus, ethanol presence during test reduces attraction towards AM (1:100), which reinstates after some time. Larvae appear to form very short retaining memory traces, making ethanol a potential reinforcer with aversive properties.

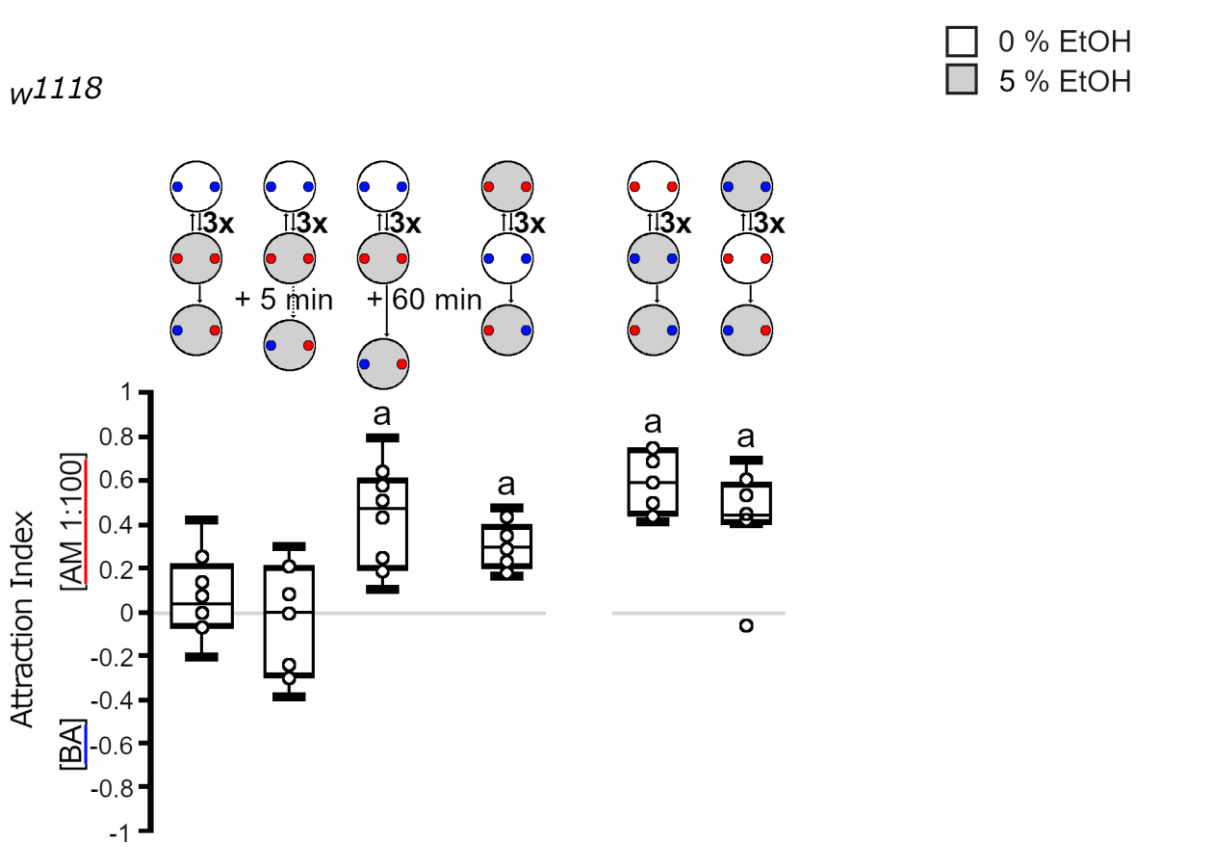

Figure 20: Ethanol presence during test reduces AM (1:100) pre-exposure attraction in a training paradigm specific manner.

Three-cycle modified and classical aversive conditioning with w^{1118} larvae and 5% EtOH reinforcement. Attraction to AM (1:100) disappears, when 5% EtOH is present during test directly and 5 minutes after conditioning but is reinstated 60 minutes after conditioning. The order of CS+ presentation is important for behavioural outcome with EtOH reinforcement in an odorant specific manner. N = 9, 10, 10, 15; 13, 11. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

w^{1118} larvae show similar behavioural patterns with 5% and 10% ethanol

5 % ethanol, which is attractive as an odorant and is not avoided by w^{1118} as a substrate, is a reinforcer with very limiting influence on overall conditioning behaviour to the larvae. Therefore, another approach was chosen to gain further insight into the role of ethanol as a potential reinforcer.

To analyse the relationship between ethanol and w^{1118} larvae further, conditioning experiments were performed with 10% EtOH reinforcement, which was aversive to w^{1118} larvae (Figure 13, Figure 15).

Strikingly, the obtained results from those conditioning experiments show a very similar picture to 5% EtOH reinforcement. If BA is paired with 10% EtOH in the second step, w^{1118} significantly avoids BA in tests on pure plates (Figure 20A). When AM (1:100) is paired with 10 % EtOH in the second step of a conditioning cycle, larvae show no preference to either odorant during test on pure plates. Pairing an odorant with 10% EtOH in the first step of a conditioning cycle, resulted in significant approach of AM (1:100) after training, suggesting that here the order of CS+ presentation is important for behavioural outcome.

To investigate this behavioural pattern further, aversive conditioning was performed (Figure 20B). Like 5% EtOH reinforcement aversive conditioning, larvae show preference for AM (1:100) on EtOH containing test plates after conditioning.

Taken together, larvae seem to show no significant behavioural changes after conditioning with ethanol, regarding the concentration of ethanol. Rather, the time-point and odorant encountered during EtOH reinforcement influences behaviour.

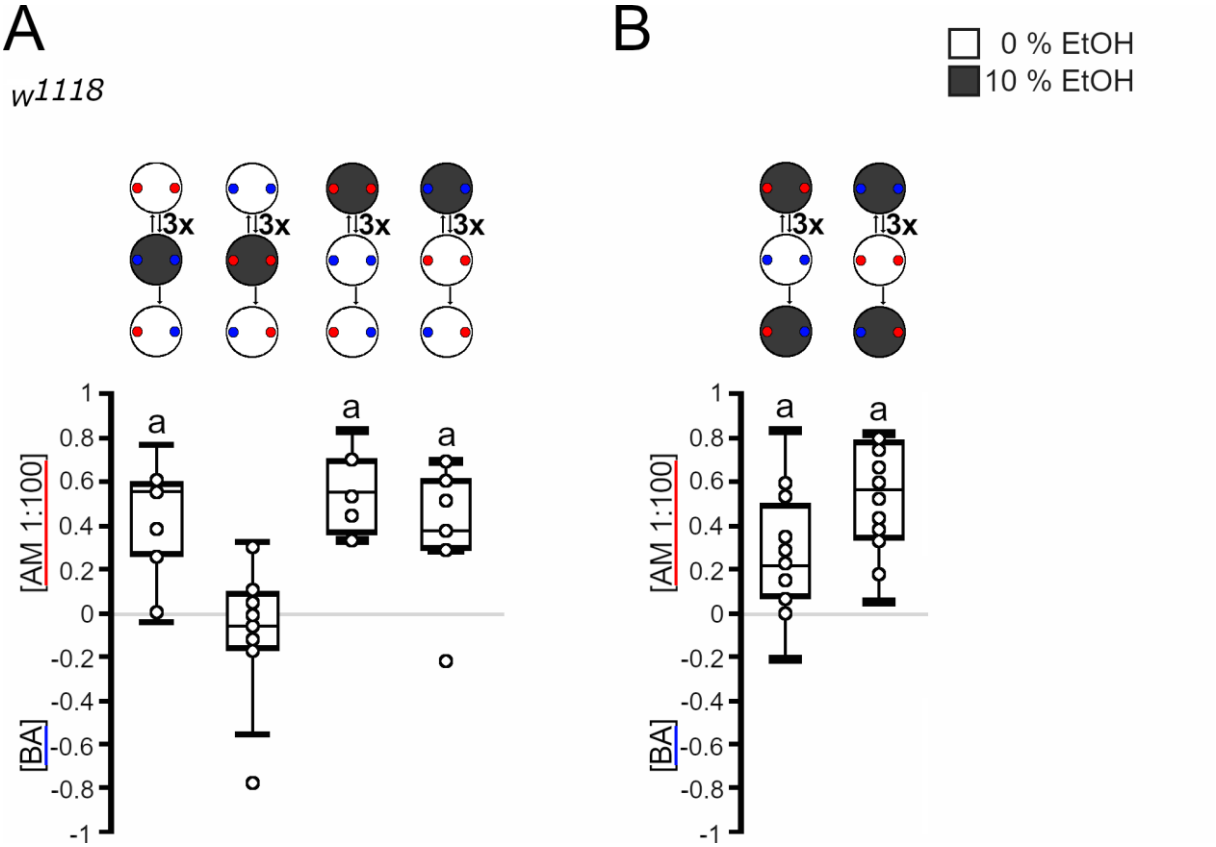

Figure 21: Larvae show odorant and trainings paradigm specific behaviour with 10% EtOH reinforcement that is like low-dose EtOH reinforcement.

A, three-cycle appetitive modified and classical conditioning was used with 10% EtOH and *w*^{1118} larvae. If AM (1:100) is paired with 10% EtOH in the second step of a conditioning cycle attraction to AM (1:100) is completely gone, however, they do not show attraction towards BA (pure). N = 11, 11, 11, 11. **B**, three-cycle aversive modified and classical conditioning was used with 10% EtOH and *w*^{1118} larvae. AM (1:100) is attractive to *w*^{1118} larvae, independent of which odorant was reinforced with 10% EtOH in step one of a conditioning cycle. N = 18, 14. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05.

Larvae prefer novel odorants to familiar odorants

Larval behaviour is regulated more by the order when the reinforce is paired with the odorant and the odorant that is paired with ethanol than by ethanol itself.

Drosophila shows preference for novel odorants (Hattori et al., 2017). Because of this, a novel odorant (2-Hep 1:300) was introduced during the test, either to exchange the CS- on EtOH test plates or the CS+ on agarose.

First, odorant acuity experiments were performed to verify that 2-Hep (1:300) as a novel odorant is not significantly more attractive during naïve encounter than the other

used odorants (Table 7). Indeed, all odorants are equally attractive, independent of ethanol concentration in the agarose plates.

Table 7: Odorant Balance experiments with *w*^{1118} for novel odorant experiments

				Odorant Balance
	AM (1:100) v 2-Hep (1:300)	**AM (1:100) v 2-Hep (1:300)**	**BA (pure) v 2-Hep (1:300)**	**BA (pure) v 2-Hep (1:300)**
Genotype				
	pure	**5% EtOH**	**pure**	**5% EtOH**
w^{1118}	**0.06 ± 0.03**	**-0.06 ± 0.07**	**0.11 ± 0.10**	**0.05 ± 0.08**
	n.s.	**n.s.**	**n.s.**	**n.s.**
N	**18**	**8**	**8**	**8**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05.

As expected, larvae show significant attraction to the novel odorant, in all tested cases (Figure 22A, 22B). It was expected that AM (1:100) as CS- is attractive to *w*^{1118}. However, the novel odorant appears to be even more attractive, overwriting the attraction towards AM (1:100). That larvae avoid BA (pure) after repeated exposure, especially in the presence of a novel –and interesting- odorant, is not surprising at all. Taken together, novel odorants are highly attractive and reveal that EtOH reinforcement might be neither attractive nor aversive but that the coupling of odorant with ethanol might be the reason for the observed larval behaviour during test.

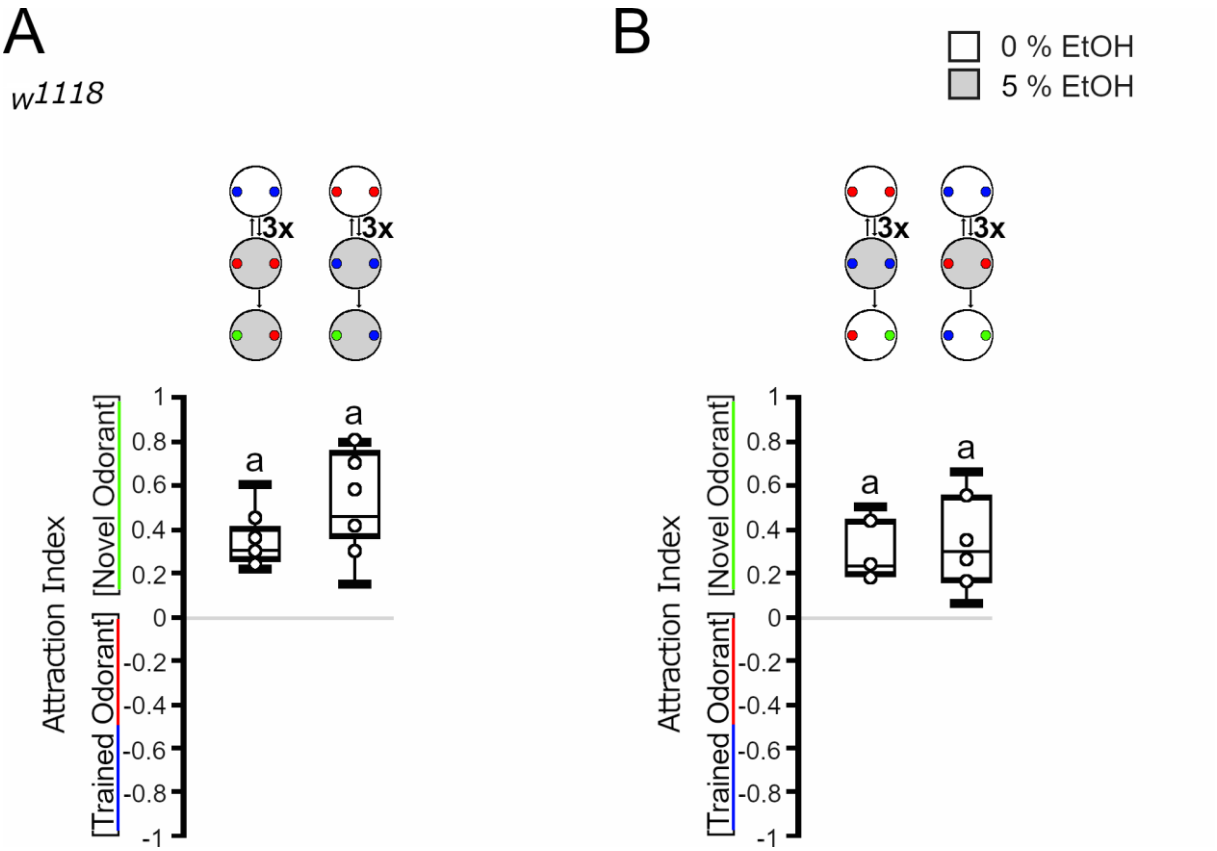

Figure 22: Novel odorants are always attractive to larvae.

A, three-cycle modified aversive training with CS- exchanged for 2-Hep (1:300) during test. Larvae of w^{1118} show significant attraction to the novel odorant, in both reciprocal groups. N = 7, 7. **B**, three-cycle classical appetitive training with CS+ exchanged for 2-Hep (1:300) during test. Larvae of w^{1118} show significant attraction to the novel odorant, in both reciprocal groups. N = 9, 9. Difference from random choice was calculated with the one sample t-test. "a" above a box plot indicates p-values <0.05.

CantonS show similar behaviour as w^{1118} after conditioning

CantonS larvae are attracted to 8% EtOH as an odorant, show decreased odorant approach on ethanol containing substrate and show attraction towards ethanol containing agarose (Figure 13, 14, 15).

To investigate the role of ethanol as a reinforcer for *Drosophila* larvae, conditioning experiments were performed with *CantonS* larvae (Figure 23). In classical appetitive, conditioning experiment *CantonS* shows attraction of AM (1:100), with and without 8% EtOH pairing during three-cycle conditioning (Figure 23A). Furthermore, if the odorant was supplemented with 8% EtOH (added to the CS+) or 8% paraffin (added to the CS-), larvae still show significant approach of AM (1:100) during training. This suggests that *CantonS* larvae can distinguish between an odorant and an odour mix and do not

interpret the odorant ethanol mix as a completely novel odorant. Modified appetitive conditioning where the reinforcer was presented during the first step of conditioning shows that larvae prefer the CS+ in both cases, which is different to results produced with w^{1118}.

It appears as if *CantonS* forms short-lived attraction towards BA (pure) under this modified conditioning paradigm.

Next, aversive conditioning experiments with *CantonS* larvae were performed (Figure 23B). When BA (pure) was paired with 8% EtOH and EtOH was present in the test plate, larvae avoided BA (pure) during test. In case of AM (1:100) supplementation with 8% EtOH and EtOH presence during test, *CantonS* also shows indifference, like w^{1118}. Interestingly, classical aversive conditioning with AM (1:100) as CS+ resulted in indecision, which was different to w^{1118}. However, these results fit with Figure 17 where *CantonS* larvae show the tendency to avoid AM (1:100) mixed with EtOH in the dot plots. The fact that BA (pure) paired with 8% EtOH during test results in aversion of BA (pure), is also consistent with the results in Figure 17.

When the CS+ is exchanged for 2-Hep (1:300) during test, *CantonS* show significant attraction towards the novel odorant, which also fits with w^{1118} results. Prior to the experiments presented in Figure 23C, odorant acuity was established to verify that neither odorant was more attractive in a naïve encounter (Table 8).

Table 8: Odorant Balance experiments with *CantonS* for novel odorant experiments

				Odorant Balance
	AM (1:100) v 2-Hep (1:300)	**AM (1:100) v 2-Hep (1:300)**	**BA (pure) v 2-Hep (1:300)**	**BA (pure) v 2-Hep (1:300)**
Genotype				
	pure	**5% EtOH**	**pure**	**5% EtOH**
CantonS	**0.02 ± 0.06**	**0.14 ± 0.09**	**-0.05 ± 0.07**	**-0.26 ± 0.11**
	n.s.	**n.s.**	**n.s.**	**n.s.**
N	**8**	**8**	**8**	**8**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05.

Taken together, *CantonS* behaves like w^{1118} after conditioning experiments.

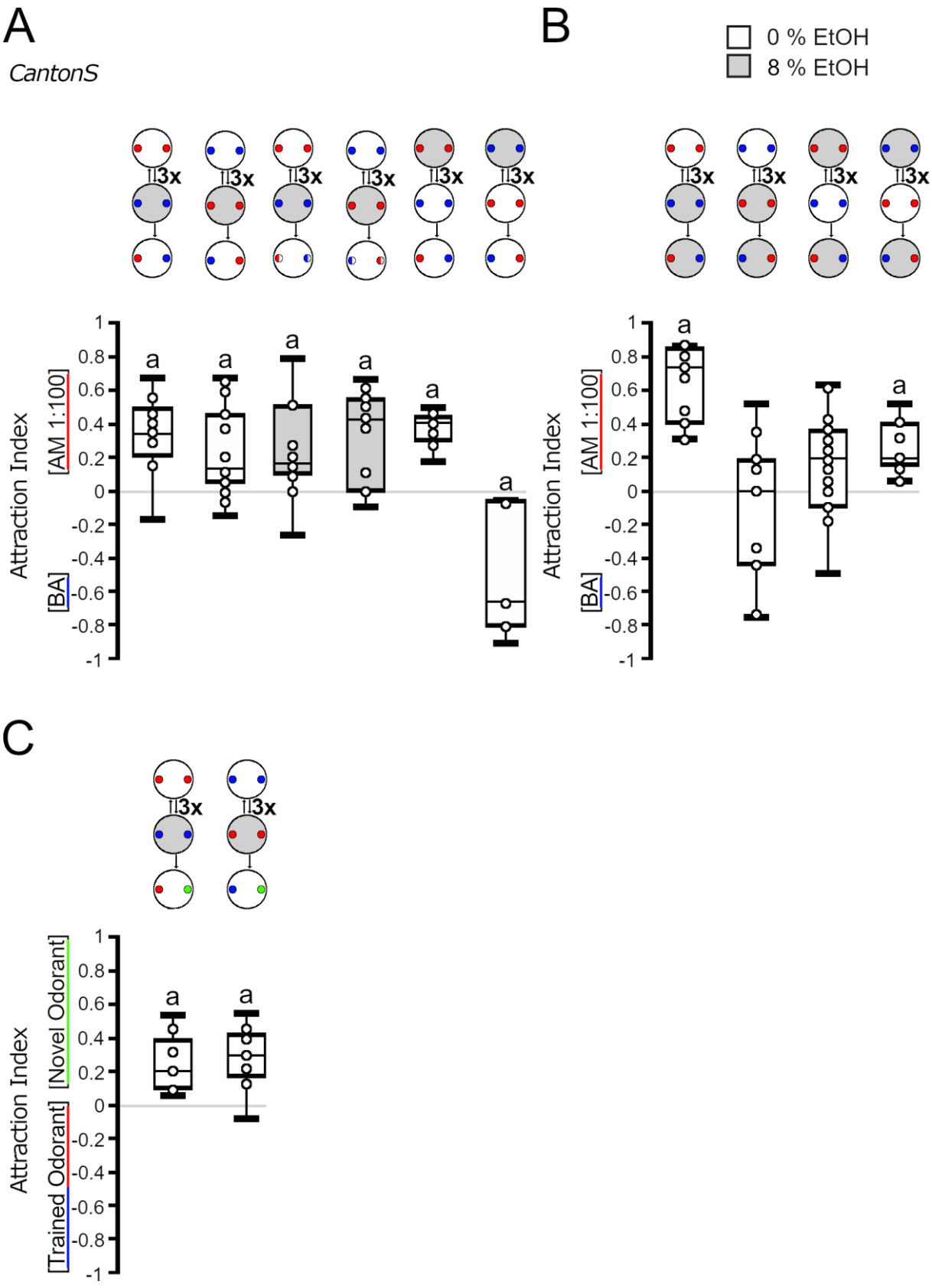

Figure 23: *CantonS* behaves like w^{1118} in olfactory conditioning experiments with EtOH

A, three-cycle classical and modified appetitive, conditioning experiments in *CantonS* larvae with appetitive 8% EtOH. *CantonS* shows similar behaviour to w^{1118} larvae after olfactory conditioning. N = 16, 16, 8, 8, 10, 10. **B**, N = 11, 11, 15, 15. **C**, N = 9, 11. Difference from random choice was calculated with the one sample t-test.

In conclusion, larvae live on ethanol and appear to be able to use ethanol as a positive teaching signal (Geer et al., 1985; Milan et al., 2012; Schumann et al., 2021). Systematically analysing olfactory conditioning with ethanol reveals that it might play less of a role as originally anticipated under the tested conditions. *Drosophila* only barely show behavioural adaptation after conditioning with ethanol and only under odorant or paradigm specific parameters. Overall short-lived behavioural adaptations appear to be indeed caused by the presence of ethanol, but in general it appears that larvae do not form memory with ethanol reinforcement. Especially the novelty odorant experiments (Figure 22, 23) are very interesting, because they support the observation that ethanol encounter during conditioning modifies behaviour under certain specific conditions but does not lead to the formation of appetitive or aversive memory in *Drosophila melanogaster* larvae.

More and further experiments need to be performed to really unravel the complex relationship between *Drosophila* larvae and ethanol, especially regarding learning and memory formation.

The role of octopamine in the internal-state and memory plasticity

Keeping track of an individual's internal state ensures the survival of the individual and thus its reproduction success (Friedman, 1997; Hardie et al., 2012; Mayer and Thomas, 1967). Therefore, the internal state must have the ability to influence animal behaviour, such as learning and memory or food consumption (Devineni and Scaplen, 2022; LeDue et al., 2016; Yang et al., 2015). Octopamine, a neurotransmitter and reported homolog for mammalian noradrenaline is known to regulate the internal-state and memory formation (Das et al., 2014; Huetteroth et al., 2015; Iliadi et al., 2017; Li et al., 2016; Scheiner et al., 2014; Schwaerzel et al., 2003; Yang et al., 2015).
Furthermore, $T\beta h^{nM18}$ mutants are characterised as null-mutants, unable to synthesize octopamine and it was reported that they show appetitive and aversive memory defects (Iliadi et al., 2017; Monastirioti et al., 1996; Schwaerzel et al., 2003). Interestingly, reports about the inability of $T\beta h^{nM18}$ mutants to form appetitive memory vary significantly, showing both ability and inability to form appetitive memory 2 minutes after training (Das et al., 2014; Huetteroth et al., 2015; Schwaerzel et al., 2003; Wu et al., 2013). This thesis aims to further clarify the role of octopamine in the formation of appetitive memory and the role of the internal state in behavioural modification.

Starvation and reinforcer strength influence appetitive 2-minute memory

To investigate the influence of the internal-state on 2-minute memory and learning performance of w^{1118} and $T\beta h^{nM18}$ mutant flies, animals were starved for 16 or 40 h prior to the experiment. Appetitive olfactory conditioning was utilised to test for memory directly after training (Figure 24). In addition, consumption behaviour was used as an additional approach to study internal-state dependent behavioural adaptation.
Prior to olfactory conditioning, adult flies were tested for their odorant acuity to verify that the used odorants, 3-Oct (1:100) and MCH (1:80) were perceived by the flies and that neither odorant was more averse or attractive than the other (Table 9). Furthermore, starvation should not influence, how the odorants are perceived and evaluated by the flies. Additionally, sucrose approach was analysed to determine the attractiveness of the used sucrose reinforcer concentration.

Table 9: Odorant acuity and control experiments with w^{1118} and $T\beta h^{nM18}$

		Reinforcer attraction		Odorant avoidance		Odorant balance
Genotype	**Starvation time**	**0.15 M sucrose approach**	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
w^{1118}	**16 h**	**0.35 ± 0.09**	**0.31 ± 0.06**	**-0.35 ± 0.09**	**-0.30 ± 0.08**	**-0.07 ± 0.06**
		*****	*******	******	******	**n.s.**
w^{1118}	**40 h**	**0.29 ± 0.06**	**0.36 ± 0.1**	**-0.58 ± 0.10**	**-0.37 ± 0.15**	**0.03 ± 0.15**
		******	*****	*******	*****	**n.s.**
$T\beta h^{nM18}$	**16 h**	**0.21 ± 0.05**	**0.31 ± 0.07**	**-0.65 ± 0.04**	**-0.68 ± 0.06**	**0.08 ± 0.16**
		******	*****	*******	*******	**n.s.**
$T\beta h^{nM18}$	**40 h**	**0.23 ± 0.05**	**0.17 ± 0.04**	**-0.32 ± 0.08**	**-0.36 ± 0.08**	**-0.06 ± 0.12**
		******	*****	*****	******	**n.s.**
N (column top to bottom)		**6/7/10/9**	**13/6/8/6**	**12/10/6/6**	**15/10/6/7**	**12/10/7/8**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Indeed, sucrose was attractive independent of starvation time and offered concentration. Furthermore, all used odorants were aversive and equally attractive or aversive in olfactory balance experiments, independent of starvation time.

To analyse the influence of the internal state of flies, appetitive olfactory conditioning experiments were performed (Figure 24A). w^{1118} flies form low, but significant, appetitive memory using 0.15 M sucrose reinforcer when starved for 16 h. When w^{1118} flies are starved for 40 h, 0.15 M reinforced appetitive memory scores are significantly higher in comparison to 16 h starved flies. Contrary to the w^{1118} control, $T\beta h^{nM18}$ mutant flies show significant aversive memory with 0.15 M sucrose reinforcement. Increased starvation leads to appetitive memory 2-minutes after training. Thus, hunger increases memory strength in w^{1118} and leads to a different form of memory in $T\beta h^{nM18}$ mutants.

To determine, whether 40 h starvation would also alter memory forms with 2 M sucrose reinforcement, appetitive olfactory conditioning experiments were performed with 2 M sucrose as reinforcer.

Here, w^{1118} shows a robust memory performance after appetitive olfactory conditioning with 2 M sucrose after 16 h starvation. These memory scores increase significantly when w^{1118} is starved for 40 h. In contrast, $T\beta h^{nM18}$ mutant flies show indifference after 2 M reinforced appetitive conditioning at 16 h starvation. When $T\beta h^{nM18}$ mutant flies starve for 40 h prior to conditioning, they can form appetitive memory 2 minutes after training. Therefore, $T\beta h^{nM18}$ can form appetitive 2-minute memory depending on the concentration of the reinforcer and the length of starvation.

In parallel to olfactory conditioning experiments, the importance of the internal state was also investigated for consumption by utilising the CAFE assay (Figure 24B). The data set presented in Figure 24B was produced by Jan Götz in 2017. The CAFE assay was performed by Götz as described in the material and methods section of this thesis. Additionally, Götz also tested 0.15 M sucrose solution and 2 M sucrose, which is the standardised reinforcer concentration for sucrose with olfactory conditioning experiments.

His results show that 16 h starved w^{1118} flies consume significantly more than $T\beta h^{nM18}$ mutant flies. However, this changes after 40 h starvation, when $T\beta h^{nM18}$ mutant flies consume significantly more in 3 h. Interestingly, in the experiments with 2 M sucrose solution, both 16 h and 40 h starved w^{1118} flies consume significantly more than $T\beta h^{nM18}$ mutant flies. This might be due to the viscosity of the 2 M sucrose solution and the decreased ability of $T\beta h^{nM18}$ mutant flies to perform in negative geotaxis assays overall (Li et al., 2016).

Thus, the internal state, represented by the starvation length, of fruit flies is directly affecting both memory formation and sucrose consumption. Furthermore, both memory formation and consumption defects of $T\beta h^{nM18}$ mutant flies can be overcome by starvation.

To verify that the observed phenotypes of $T\beta h^{nM18}$ mutant flies at 40 h starvation are linked to changes of their internal carbohydrate storage, whole-body glycogen was measured (Figure 24C). Indeed, $T\beta h^{nM18}$ flies show significantly increased levels of overall glycogen storage, independent of starvation time. Therefore, increased glycogen storage might be the explanation for $T\beta h^{nM18}$ mutants to not exhibit appetitive STM shortly after training and consume significantly less sucrose.

Next, qPCR was performed to analyse, if the increased levels of glycogen in $T\beta h^{nM18}$ flies are due to an up- or downregulation of either *GlyP* or *GlyS* (Figure 24D). These results were produced by Evelin Fahle in 2022. cDNA of w^{1118} and $T\beta h^{nM18}$ mutants

was extracted with a standardised protocol. First total RNA was extracted from both w^{1118} and $T\beta h^{nM18}$ mutant male flies. RNA concentration was determined with the nanodrop photometer. Based on this, Fahle performed cDNA synthesis, following an established protocol in the lab with the Superscript II Kit bought from Invitrogen. A qPCR was performed by Fahle. As reference sample the *RpLP0* gene was used, which encodes for the RpLP0 protein, a ribosomal protein that is part of the *Drosophila* DNA repair mechanism and homologous to the human P0 protein (Grabowski et al., 1991; Yacoub et al., 1996). The gene expression ratios were analysed with an Excel sheet provided by TopTipBio.com (last downloaded on 05.04.2022) with the Pfaffl-method.
Interestingly, neither *GlyP* nor *GlyS* was up- or downregulated in $T\beta h^{nM18}$ flies in comparison to w^{1118} (Figure 24D), which would have provided a potential explanation for the increased glycogen storage in $T\beta h^{nM18}$ mutant flies.
Taken together, it can be said that both, the reinforcer strength, and the internal state, are directly influencing memory formation and carbohydrate consumption in w^{1118} and $T\beta h^{nM18}$. Strikingly, $T\beta h^{nM18}$ mutants can show memory 2 minutes after conditioning and the reinforcer strength determines whether flies form aversive or appetitive memory to a sugar reward.

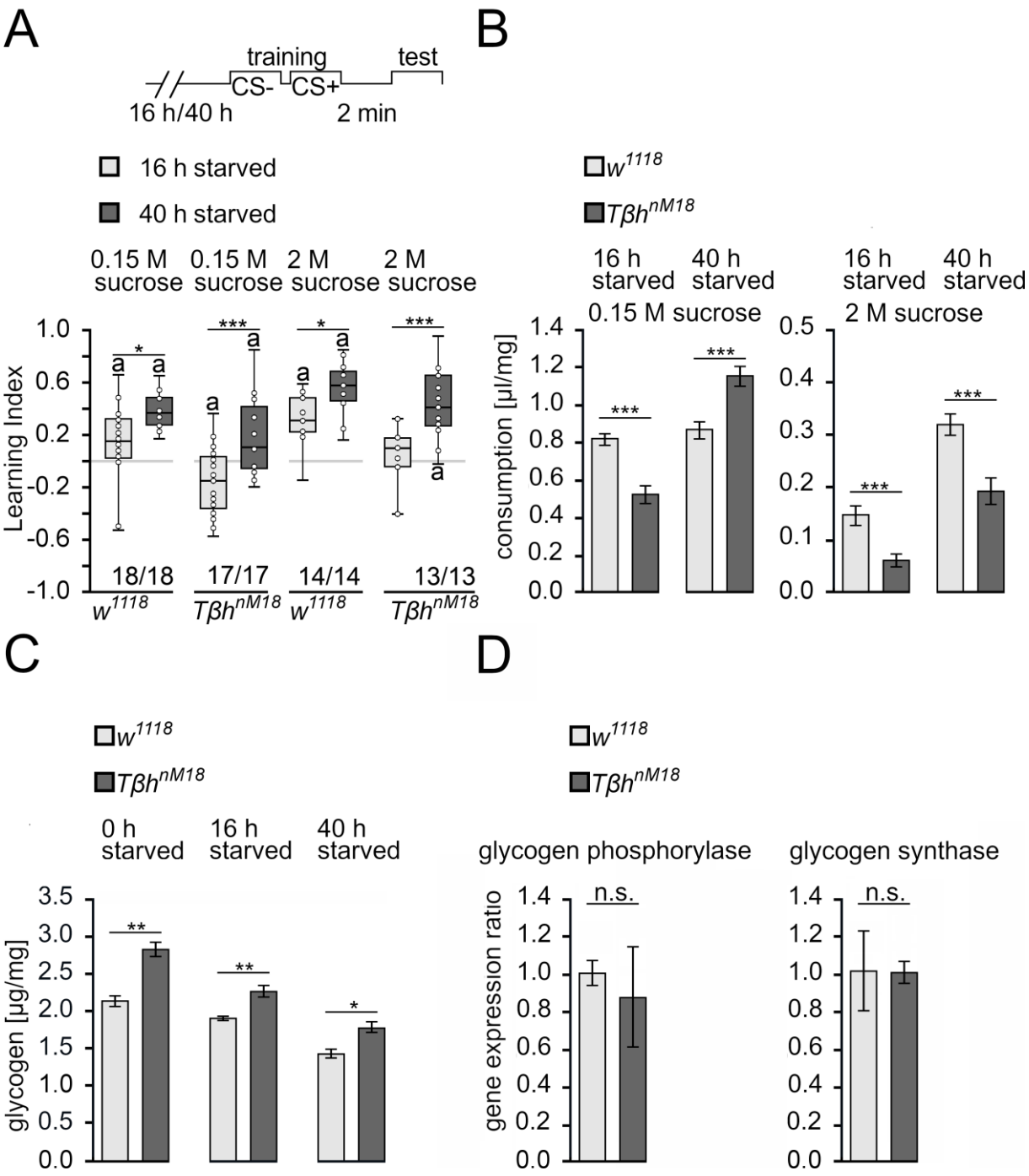

Figure 24: The internal state regulates appetitive memory formation in *w*^{1118} and *Tβh*^{nM18} mutants. **A**, appetitive STM training with 0.15 M and 2 M sucrose produces appetitive STM in *w*^{1118} flies, which significantly increases upon 40 h starvation. *Tβh*^{nM18} mutant flies form aversive STM with 0.15 M sucrose and no memory with 2 M sucrose. 40 h starvation significantly increases appetitive memory under both tested reinforcer concentrations. N = 18, 18; 17, 17; 14, 14; 13, 13. **B**, (Data set produced by Jan Götz, 2017) 3 h CAFE assay with 0.15 M and 2 M sucrose under 16 h and 40 h starvation conditions. *w*^{1118} flies consume significantly more sucrose than *Tβh*^{nM18} mutant flies when starved for 16 h prior to consumption experiments with 0.15 M and 2 M sucrose. Increased starvation of 40 h leads to overconsumption in *Tβh*^{nM18} mutant flies with 0.15 M sucrose but not with 2 M sucrose. N = 20, 20, 20,

20; 18, 18, 18, 18. **C**, whole-body glycogen content in w^{1118} and $T\beta h^{nM18}$ mutant flies under 0 h, 16 h and 40 h starvation conditions. Under all measured starvation conditions $T\beta h^{nM18}$ mutants have significantly more overall stored glycogen. N = 3, 3; 3, 3; 3, 3. **D**, (Data set produced by Evelin Fahle, 2022) *GlyP* and *GlyS* expression ratio in w^{1118} and $T\beta h^{nM18}$ mutant flies. No significant differences of *GlyP* or *GlyS* were observed with qPCR N = 3, 3; 3, 3.
Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student’s t-test. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

The internal state influences memory stability

Drosophila melanogaster flies form appetitive memory after STM conditioning. Although, $T\beta h^{nM18}$ mutants require increased starvation (this thesis), or increased reinforcer strength to show memory 2 minutes after training (Huetteroth et al., 2015). However, what form of memory they exactly exhibit, is yet not clear. Therefore, further conditioning experiments were performed that tested memory decay under different starvation conditions as well as anaesthesia-resistance of those memory performances (Figure 25).
After one-cycle of appetitive olfactory conditioning with w^{1118}, flies show no significant memory 6 h after training, when starved for 16 h hours (Figure 25A). When the starvation time is increased, 40 h, w^{1118} shows significant and stable memory 6 h after training. Interestingly, $T\beta h^{nM18}$ show stable and significant memory 6 h after training, independent of their starvation period prior. 16 h and 40 h starved 6-h memory is not significantly different from each other in $T\beta h^{nM18}$ mutant flies.
To ascertain whether starvation not only changed memory stability but also the memory form in w^{1118} and $T\beta h^{nM18}$ cold-shock experiments were performed. These experiments also allowed to further dissect the specific memory (Eschment et al., 2020; Krashes and Waddell, 2008) that $T\beta h^{nM18}$ mutant flies form, which appears to be independent of the internal-state hunger (Figure 25B).
Rapidly consolidated protein-synthesis dependent memory was already reported in flies with high reinforcer concentrations (Krashes and Waddell, 2008). There, an applied cold-shock shortly after training significantly disrupted memory that was protein-synthesis dependent and thus in need of memory consolidation (Krashes and Waddell, 2008). This was used to differentiate between protein-synthesis independent ARM and protein-synthesis dependent LTM (Figure 25B). Cold shock was either applied directly afterwards or 2 h after the training, which is used to test for ARM (Tully

et al., 1994). Consolidated memory is cold shock resistant in both ARM and LTM, which is why directly applied cold shock disrupts consolidation and protein-synthesis dependent forms of memory.

w^{1118} flies show low appetitive memory 3 h after training, which was significantly different from random choice. Thus, memory after one-cycle training is not just 2-minute short-term memory but lasts for longer periods of time. However, w^{1118} flies that starved for 16 h show no cold shock resistant memory 3 h after training. Interestingly, $T\beta h^{nM18}$ mutants also show memory 3 h after training, which could be erased by cold-shock directly after training but not 2 h after training. This suggests, that $T\beta h^{nM18}$ mutants form, internal-state independent consolidation dependent appetitive memory. In summary, when w^{1118} flies are starved for 40 h before training, one-cycle conditioning with 2 M sucrose reinforcement produces strong and significant appetitive memory 3 h that can be erased by cold shock directly after training but not 2 h after training. Thus, increased starvation shifts memory in w^{1118} from STM to LTM. $T\beta h^{nM18}$ mutant flies show a different form of memory as well, when they are starved for 40 h prior to training. Here, their memory could not be erased by cold-shock shortly after or 2 h after training. This suggests, that $T\beta h^{nM18}$ mutants can form internal-state dependent long lasting memory that is rapidly consolidated.

Taken together, these results provide insight into the influence of the internal state on expressed memory forms as well as how lack of octopamine in $T\beta h^{nM18}$ mutant flies results in exclusively appetitive consolidation dependent memory.

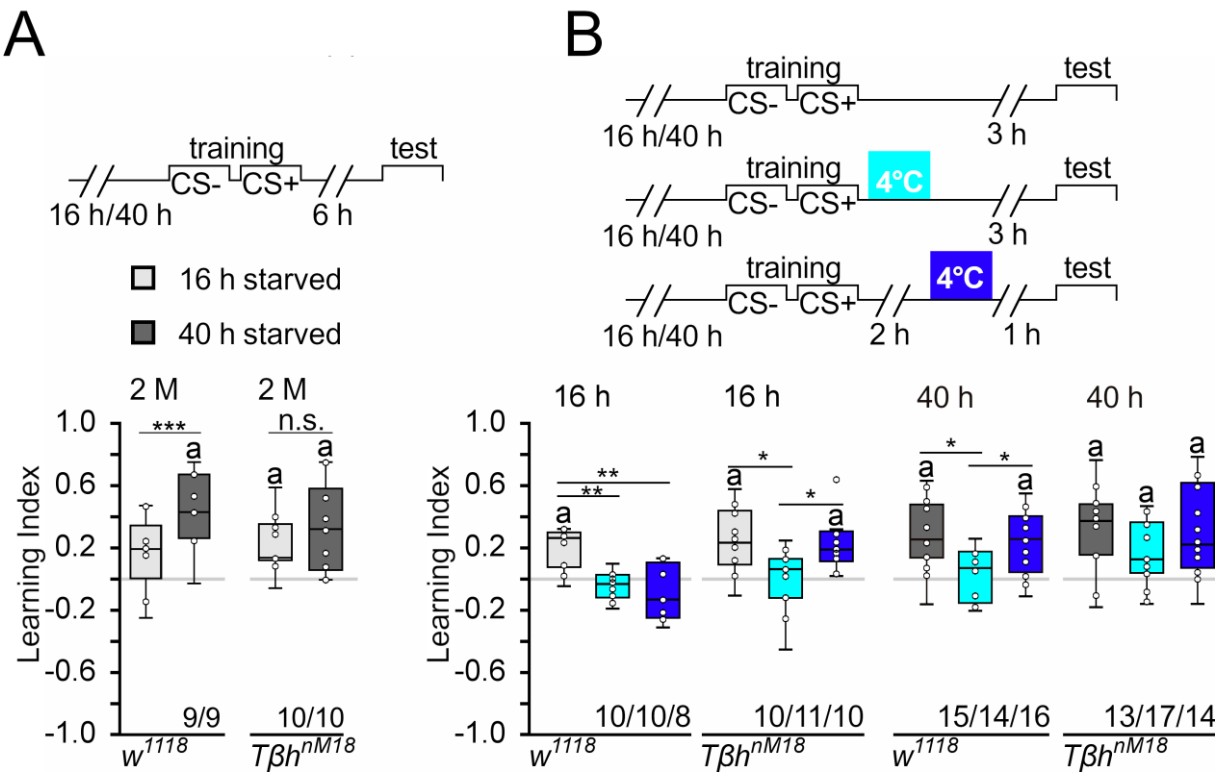

Figure 25: Increased starvation results in increased stability of appetitive memory in both w^{1118} and $T\beta h^{nM18}$ mutants

A, appetitive olfactory conditioning with 16 h and 40 h starved w^{1118} and $T\beta h^{nM18}$ flies with 2 M sucrose reinforcement. 6 h after training 16 h starved w^{1118} flies show indecision after STM conditioning, while $T\beta h^{nM18}$ flies show memory. 40 h starved w^{1118} and $T\beta h^{nM18}$ flies both show appetitive memory 6 h after training, that is not significantly different from each other. N = 9,9; 10, 10. **B**, cold-shock experiments after STM conditioning with 16 h and 40 h starved w^{1118} and $T\beta h^{nM18}$ flies. Cold shock was applied 2 minutes after training or 2 hours after training. 16 h starved w^{1118} flies show memory 3 h after STM training, that can be erased by cold-shock anaesthesia, while $T\beta h^{nM18}$ fly 3 h memory is still present when cold shock is applied after the consolidation phase. 40 h starved w^{1118} memory is significantly more stable and cannot be erased by cold shock applied 2 h after training, while $T\beta h^{nM18}$ memory is even more stable and cannot be erased by cold shock at all. N = 10, 10, 8; 10, 11, 10; 15, 14, 16; 13, 17, 14. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student’s t-test. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

$T\beta h^{nM18}$ mutant flies form protein-synthesis dependent appetitive memory

$T\beta h^{nM18}$ mutants form an internal state independent memory that lasted for at least 6 h, which is strongly suggested to be protein-synthesis dependent LTM. However, this

has to be further analysed by addressing specific dopaminergic neurons that are responsible for appetitive LTM formation (Yamagata et al., 2015).

Therefore, to investigate the specific memory formed in *Tβh*^{nM18} mutants, heat shock experiments with *shibire*^{ts} were performed to specifically address neurons that are reported to be responsible for appetitive LTM (*R15A04-Gal4*) (Huetteroth et al., 2015; Yamagata et al., 2015). Those experiments were performed by Katrin Auweiler. Fly crosses were raised on 18°C to inhibit the effect of *shibire*^{ts} during development. Flies were starved for 16 h prior to experiments and one-cycle STM appetitive conditioning was performed at 20°C with 2 M sucrose reinforcement. This training was expected to result in short-term memory. Directly after training the groups received a heat shock in an incubator at 31°C for 30 min, to mimic consolidation inhibition through cold shock (Figure 25B) and were transferred to 20°C for the rest of the time afterwards. The test was performed 6 h after training.

The R15A04-Gal4 heterozygous control did not show appetitive memory 6 h after training (Figure 26), which fits with the observations of *w*^{1118} flies not being able to retain appetitive memory after one-cycle training (Figure 25A). Both *Tβh*^{nM18} genetic background control groups show appetitive memory after one-cycle training, which is also in accordance with the results in Figure 25. However, the experimental group did not show any significant memory after heat shock. This shows, that *Tβh*^{nM18} mutant flies form internal-state independent appetitive LTM.

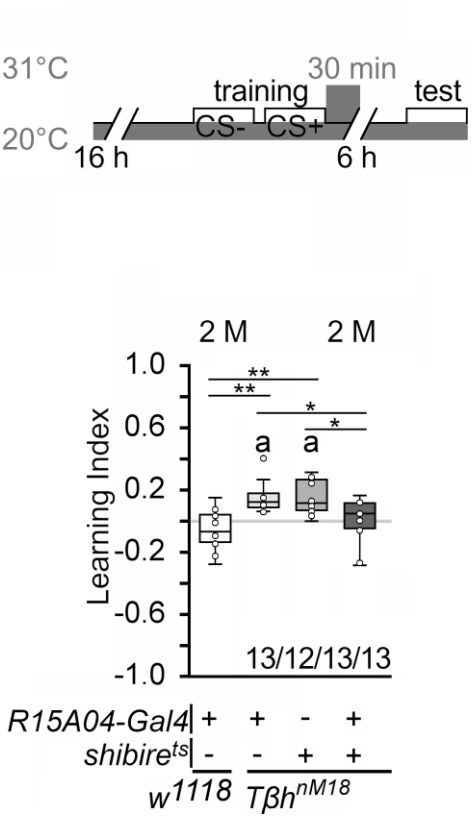

Figure 26: *Tβh^{nM18}* mutants form LTM

(Data set produced by Katrin Auweiler, 2022.) One-cycle appetitive learning experiments with 2 M sucrose with *shibire^{ts}* blocked DA *R15A04-Gal4* targeted neurons in the *Tβh^{nM18}* mutant background. Heat shock was applied after conditioning for 30 min. Test was performed 6 h later. Blocking signalling through DANs resulted in no significant memory in flies with the *Tβh^{nM18}* mutants. Furthermore, the obtained results for the experimental group did not differ from the genetic controls. N = 13, 12, 13, 13. Difference from random choice was calculated with the one sample t-test. "a" above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student's t-test. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Tβh^{nM18} form appetitive STM in an internal-state dependent manner

Appetitive memory can be modified by starvation (Figure 24, 25). However, while *w^{1118}* can form appetitive STM 2 min after training, which is measurable up to 3 h later, *Tβh^{nM18}* mutants show no apparent memory 2 min after training. This is because they form appetitive LTM which needs consolidation and is anaesthesia-resistant upon increased starvation. Previously it has been suggested that *Tβh^{nM18}* mutants fail to form appetitive STM (Das et al., 2014; Schwaerzel et al., 2003). To investigate the ability of *Tβh^{nM18}* mutants to form appetitive memory, olfactory conditioning experiments were

performed with female flies under different mating conditions and with male flies with another attractive reinforcer, yeast (Figure 27). Female flies were chosen as another option to analyse internal-state changes in animals, because they can be mated or non-mated, which alters their internal-state (Corrales-Carvajal et al., 2016; Tian and Wang, 2018; Vargas et al., 2010).

Virgin flies of w^{1118} show appetitive memory 2 min after conditioning (Figure 27A). However, when the internal state of female flies changes after mating, this results in a significant preference for protein sources which are necessary for egg production (Vargas et al., 2010). This can explain, why mated w^{1118} female flies no longer show any STM directly after training with 2 M sucrose reinforcer. Similarly, $T\beta h^{nM18}$ virgin females also show appetitive STM with 2 M sucrose reinforcement, which is not observable, when the mutant flies are mated (Figure 27A). This strongly indicates, that $T\beta h^{nM18}$ mutant appetitive STM defects are indeed due to the internal state of the animal.

Furthermore, when $T\beta h^{nM18}$ male flies are trained with 5% yeast extract as reinforcement, they can form appetitive STM like w^{1118} flies (Figure 27B). To support the observation that the valence for nutrients shifts upon internal state changes additional control consumption experiments with 5% yeast over 24 h were performed (Figure 27C). In a normal, sated male w^{1118} consume significantly less than $T\beta h^{nM18}$, meaning that lack of octopamine results in an increased appetite for protein rich sources. This significant difference disappears after a 16 h starvation period, suggesting that under starvation conditions, both animals have equal requirements regarding proteins.

Taken together, $T\beta h^{nM18}$ can form internal state dependent appetitive STM and has an increased appetite for protein food sources.

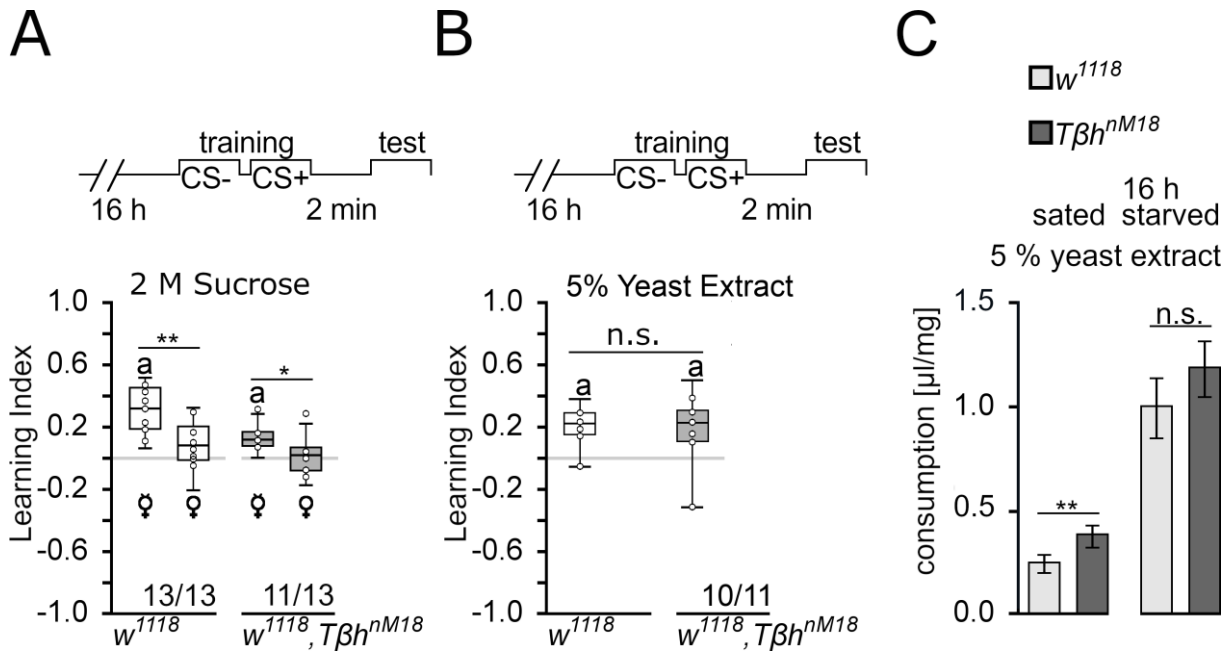

Figure 27: *TβhnM18* mutant flies can form appetitive internal-state dependent STM

A, Non-mated and mated *w1118* and *TβhnM18* female flies were tested after training with 2 M sucrose reinforcer. Virgin female *w1118* flies form robust and significant memory with 2 M sucrose reinforcement, which is not present in mated *w1118* flies. *TβhnM18* virgin female flies also form low, but significant appetitive memory, which disappears in mated *TβhnM18* flies. N = 13, 14; 11, 13. **B**, appetitive STM conditioning with 5% yeast reinforcement. Both, *w1118* and *TβhnM18* show appetitive memory after one-cycle training. N = 10, 11. **C**, 24 h consumption of 5% yeast extract with *w1118* and *TβhnM18* mutant flies, with and without starvation prior to the beginning of the experiment. In a sated state, *TβhnM18* flies consume significantly more than *w1118*. However, when the animals are starved both genotypes overconsume and are no longer significantly different from each other. N = 26, 26; 26, 26. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between two groups was calculated with the student’s t-test. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

TβhnM18 show an innate preference for protein food sources

TβhnM18 flies have significantly more appetite for yeast extract than *w1118*. However, these experiments only offered 5% yeast extract without any alternative. Thus, it would be interesting to see, how *TβhnM18* mutants’ consumption behaviour would change, when a choice situation is offered.

To analyse food choice behaviour, 24 h CAFE assays were performed with 0.15 M sucrose and 5% yeast choice (2 capillaries each) with *w1118* and *TβhnM18* (Figure 28). Interestingly, in a choice situation *w1118* and *TβhnM18* show an overall total consumption, that is not significantly different from each other (Figure 28A). When flies are starved

16 h before the experiment began, a significant overconsumption in *Tβh*^{nM18} flies was observable, that further increased when flies were starved for 40 h before the experiment.

To determine, whether *Tβh*^{nM18} overconsumption is due to sucrose or yeast, a sucrose preference was calculated (Figure 28B). These results suggest, that sated and 16 h starved *Tβh*^{nM18} mutants show significantly decreased appetite for 0.15 M sucrose. Seeing that, sated *Tβh*^{nM18} show an overall equal appetite but reduced sucrose preference, it can be said that *Tβh*^{nM18} consume more yeast in comparison to *w*^{1118}. Interestingly, *w*^{1118} and *Tβh*^{nM18} have equally reduced sucrose preference when they are starved for 40 h prior to the experiment. Therefore, the results show that starvation shifts food preference in both *w*^{1118} and *Tβh*^{nM18} flies from carbohydrates to proteins. To further investigate consumption behaviour in *w*^{1118} and *Tβh*^{nM18} flies, 24 h CAFE assays with a mix of 0.15 M sucrose and 5% yeast were performed (Figure 28C). This way, food consumption of a food source that is closer to mimicking *Drosophila* food sources in the environment could be analysed. Here, sated and 16 h starved *w*^{1118} consumes significantly more sucrose-yeast mixture than *Tβh*^{nM18} and only consume less when they are starved for 40 h prior to the experiment.

To link consumption of proteins and learning results with yeast extract reinforcement, Bradford-tests were performed to measure the protein levels of *w*^{1118} and *Tβh*^{nM18} flies by Manuela Ruppert in 2017. For this, she took 5 male flies 5 days of age that were either fed or starved for 16 h and homogenised the animals to measure protein concentration photometrically. The protocol was described previously (Bradford, 1976). Results were normalised for fly weight to circumvent differences in levels of protein due to size and weight differences between *w*^{1118} and *Tβh*^{nM18}. Here, *w*^{1118} shows significantly higher levels of protein concentration in comparison to *Tβh*^{nM18}, which fits nicely to the internal-state dependent consumption and learning phenotypes. These results suggest that sucrose in the food solution is the limiting factor for *Tβh*^{nM18} mutant consumption and that the internal state strongly regulates consumption of different nutrients in *w*^{1118} and *Tβh*^{nM18}.

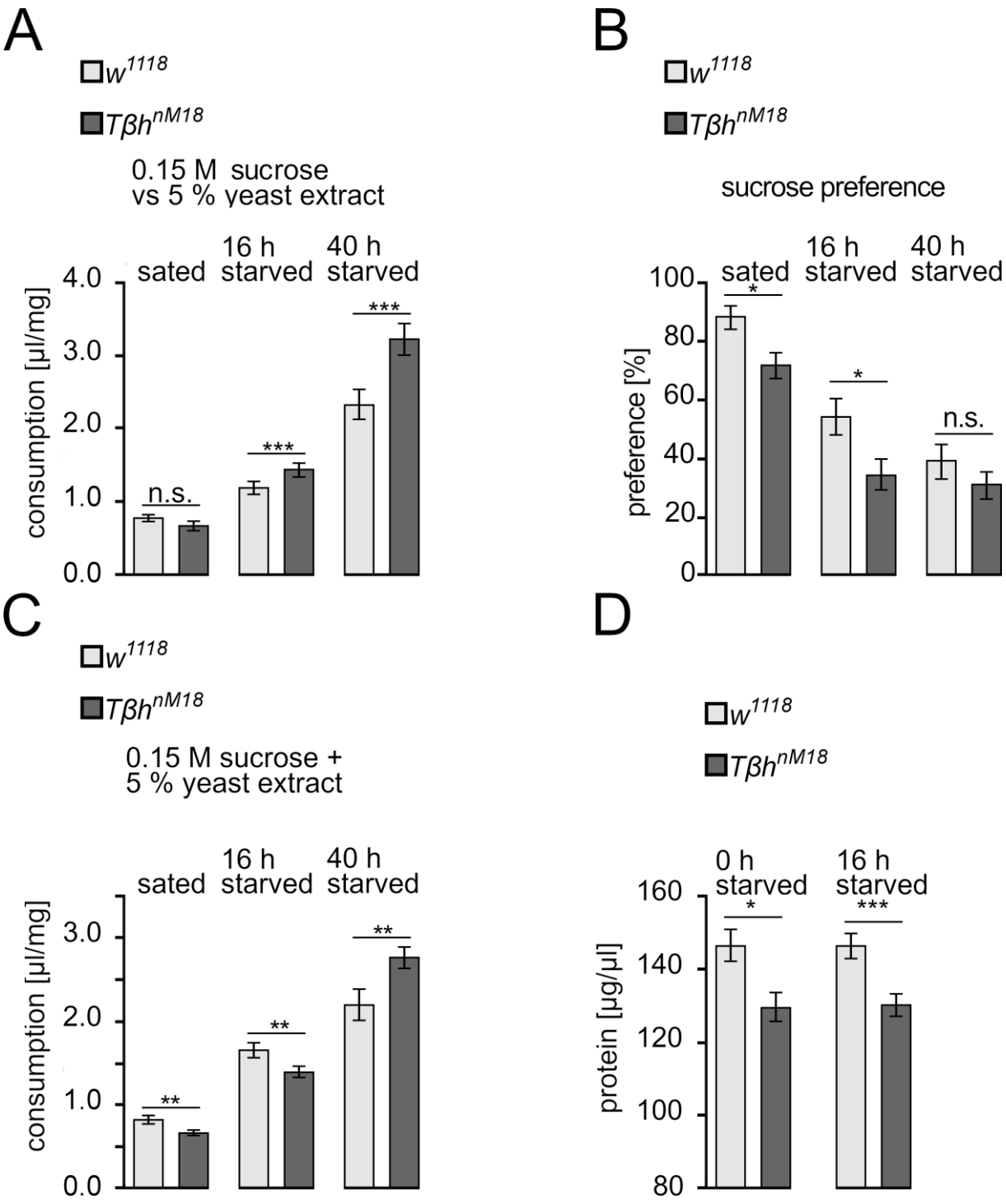

Figure 28: *Tβh^{nM18}* mutants have an increased appetite for yeast

A, 24 h CAFE assay with 0.15 M sucrose and 5% yeast in a choice situation with *w^{1118}* and *Tβh^{nM18}*. When sated the overall consumption of *w^{1118}* and *Tβh^{nM18}* mutant flies is equal. However, upon 16 h and 40 h starvation, *Tβh^{nM18}* consumes significantly more in comparison to *w^{1118}*. N = 31, 36; 35, 38; 35, 40. **B**, calculated sucrose preference from the results of Figure 28A. *Tβh^{nM18}* mutants show decreased preference for carbohydrate food sources when sated and 16 h starved prior the experimental beginning. Decreased preference for sucrose disappears in *Tβh^{nM18}* mutants in comparison to *w^{1118}* when starved for 40 h prior to experimental beginning. N = 31, 36; 35, 38; 35, 40. **C**, 24 h CAFE assay with 0.15 M

and 5% yeast extract mixed with w^{1118} and $T\beta h^{nM18}$ mutants. Sated and 16 h starved $T\beta h^{nM18}$ flies consume significantly less food solution than w^{1118}. However, 40 h starved $T\beta h^{nM18}$ mutants consume significantly more food solution. N = 26, 26; 26, 26; 21, 25. **D**, (Data set produced by Manuela Ruppert, 2017) protein levels in *Drosophila* fly homogenate measured with the Bradford-test. $T\beta h^{nM18}$ flies show significantly reduced levels of whole-body protein in comparison to w^{1118}. Starvation for 16 h does not significantly reduce protein levels in comparison to the satiated groups. N = 11,11,11,11. Difference between two groups was calculated with the student's t-test. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Muscle glycogen negatively regulates appetitive STM formation in flies

Starvation regulates food intake, which alters the internal state of *Drosophila melanogaster* flies. Upon starvation, stored resources are depleted to ensure survival in time of need (Preiss and Walsh, 1981; Roach et al., 2012). Glycogen is the main carbohydrate storage that is mostly present in *Drosophila* flight muscles, haltere, and fat body (Wigglesworth, 1949). Thus, changes of the level of glycogen storage in the fly should result in behavioural changes.

To investigate whether the level of glycogen in the flight muscles influence appetitive learning and memory, *mef2-Gal4*, a Gal4-line that expresses primarily in muscles (Viswanathan et al., 2015), was crossed with either *UAS-GlyP-RNAi* or *UAS-GlyS-RNAi* (Perkins et al., 2015; Yamada et al., 2018) to alter glycogen only in certain storage organs (Figure 29).

For visualisation purposes and as a proof that the RNAi is indeed functional, larval tissue was dissected to perform PAS staining (Yamada et al., 2018), that would provide insight into the functionality of the genetic tools (Figure 29A). Indeed, both *GlyP-* and *GlyS-RNAi* properly up- and downregulated glycogen in body-wall muscles respectively, as indicated by changes in the pink colouration.

Odorant acuity was tested for all groups mentioned in table 10 to verify the ability of flies to smell the odorants and to determine whether one of the odorants was more attractive or aversive, as well as whether the sucrose reinforcer was an appetitive stimulus to the flies (Table 10).

The reinforcer, 2 M sucrose, was attractive to the flies and all odorants were significantly avoided and balanced during simultaneous presentation.

Table 10: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (mef2)

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
mef2-Gal4/+	**0.28 ± 0.06**	**-0.46 ± 0.07**	**-0.40 ± 0.13**	**0.06 ± 0.05**
	******	******	*****	**n.s.**
UAS-GlyP-RNAi/+	**0.46 ± 0.06**	**-0.43 ± 0.04**	**-0.33 ± 0.08**	**-0.10 ± 0.06**
	*******	*******	******	**n.s.**
mef2-Gal4/GlyP-RNAi	**0.27 ± 0.07**	**-0.25 ± 0.06**	**-0.28 ± 0.06**	**-0.01 ± 0.05**
	******	******	******	**n.s.**
UAS-GlyS-RNAi/+	**0.27 ± 0.06**	**-0.37 ± 0.07**	**-0.47 ± 0.04**	**-0.15 ± 0.10**
	******	******	*******	**n.s.**
mef2-Gal4/GlyS-RNAi	**0.25 ± 0.07**	**-0.19 ± 0.04**	**-0.15 ± 0.04**	**-0.09 ± 0.08**
	******	******	******	**n.s.**
N (column top to bottom)	**7/8/9/8/8**	**6/17/9/6/8**	**6/8/8/10/8**	**8/10/8/6/8**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Next, appetitive STM conditioning using 2 M sucrose as a reinforcer to address whether flight muscle glycogen influences the level of appetitive short-term memory was performed (Figure 29B). Upregulation of flight muscle glycogen did not alter appetitive STM formation in comparison to both genetic controls. However, downregulation of flight muscle glycogen significantly increases appetitive STM in comparison to both genetic controls. Therefore, flight muscle glycogen negatively regulates appetitive STM using 2 M sucrose as reinforcer.

Next, whole-body glycogen levels were analysed in adult *Drosophila* (Figure 29C) to investigate how significant the changes of artificial glycogen storage depletion or increase would be on the organism. Surprisingly, neither upregulation through GlyP knockdown nor downregulation through GlyS knockdown significantly altered whole-body glycogen levels.

To further investigate the role of flight muscle glycogen on sucrose intake, CAFE assays were performed with 0.15 M sucrose solution (Figure 29D and 29E). Unexpectedly, neither flight muscle glycogen up- nor downregulation in 3h (Figure 29D) and 24 h (Figure 29E) CAFE assays resulted in significant changes in behaviour. Taken together, the level of glycogen in the flight muscle influences appetitive STM. Interestingly, those changes do not need to be striking. Rather, flight muscles are apparently reacting to slight changes in glycogen storage to regulate behaviour downstream.

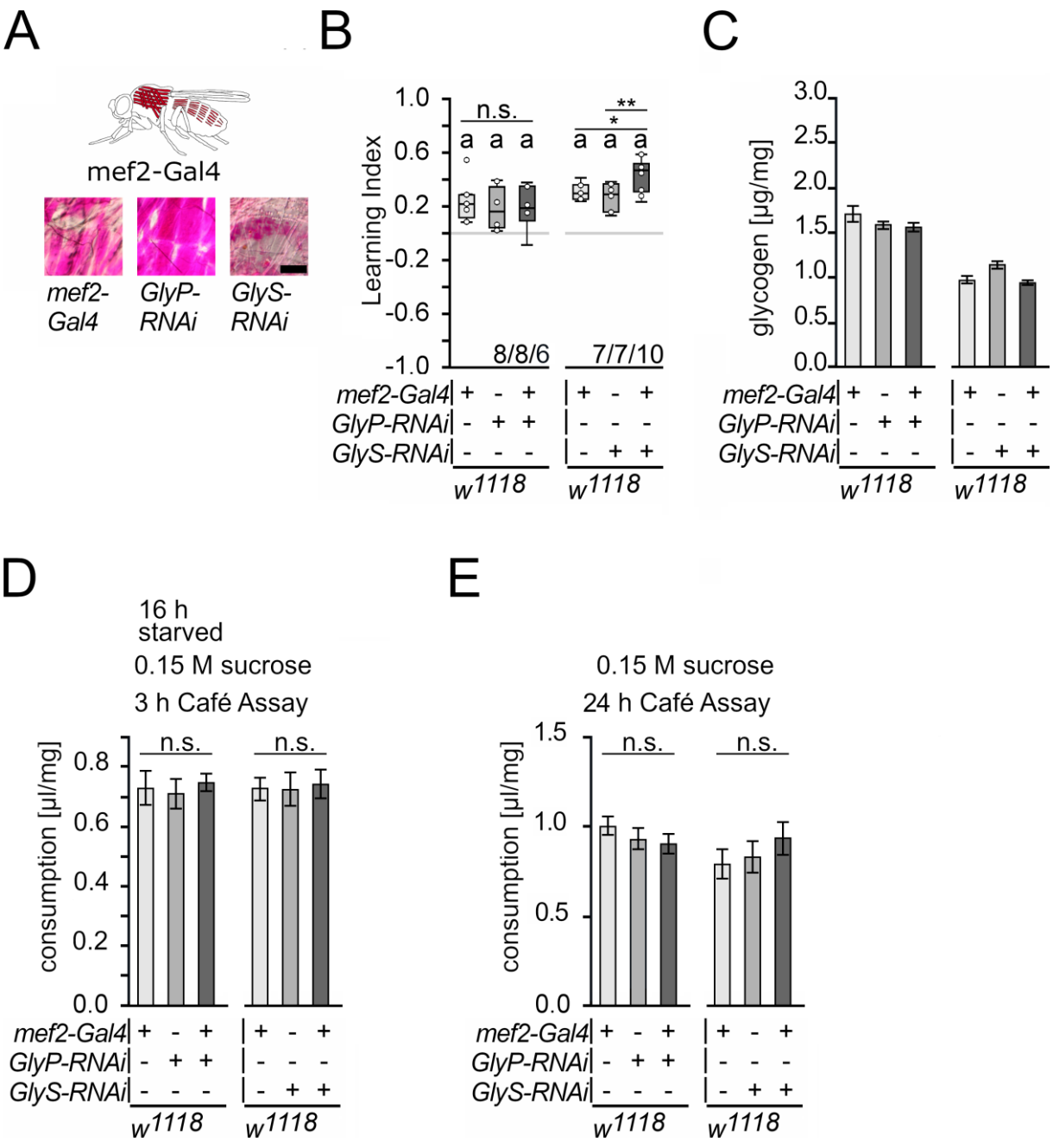

Figure 29: Muscle glycogen levels are negatively regulating appetitive STM formation but not carbohydrate consumption.

A, schematic overview of adult flight muscles and larval body-wall muscle PAS staining with RNAi mediated knockdown of *GlyP* and *GlyS.* Scale bar = 25 µm. **B**, appetitive STM with 2 M sucrose. Upregulation of glycogen in flight muscles does not influence appetitive memory formation. However, downregulation of flight muscle glycogen does negatively influence appetitive STM formation. N = 8, 8, 6; 7, 7, 10. **C**, whole-body glycogen measurements with the Glucose Assay Kit. Neither up- nor downregulation of flight muscle glycogen does significantly change whole-body glycogen in the animals. N = 3, 3, 3; 3, 3, 3. **D**, CAFE assay, measuring hunger for sucrose, with RNAi mediated up- or downregulation of flight muscle glycogen with 0.15 M sucrose. Hunger in 16 h starved animals does not influence carbohydrate consumption, when flight muscle glycogen is up- or downregulated. N = 10, 18, 18; 15, 30, 15. **E**, CAFE assay, measuring appetite, with RNAi mediated up- or downregulation of flight muscle glycogen with 0.15 M sucrose. Appetite is not altered by up- or downregulation of glycogen in the flight muscles. N = 26, 28, 30; 36, 40, 30. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Fat body glycogen storage does not influence appetitive STM

Flight muscle glycogen levels directly influence appetitive STM in *Drosophila melanogaster*. Another important glycogen storage organ is the fat body (Wigglesworth, 1949), addressed by the *FB-Gal4* line (also known as r^4*-Gal4*) (Lee and Park, 2004). Therefore, it was interesting to investigate how artificial changes to the fat body glycogen storage would affect appetitive behaviour.

To investigate the role of fat body glycogen as behavioural regulator, the *FB-Gal4* line was crossed with *UAS-GlyP-* and *UAS-GlyS-RNAi* fly lines (Perkins et al., 2015; Yamada et al., 2018) and analysed in learning and memory and sucrose intake (Figure 30).

First, olfactory acuity was analysed in the flies to determine whether they prefer the reinforcer and avoid the odorants equally (Table 11). All groups show preference towards 2 M sucrose and avoidance of the used odorants as well as balanced behaviour, when the odorants are presented simultaneously to naïve flies.

Table 11: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (FB)

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
FB-Gal4/+	**0.20 ± 0.06**	**-0.35 ± 0.06**	**-0.39 ± 0.05**	**0.05 ± 0.14**
	*****	*******	*******	**n.s.**
FB-Gal4/+;GlyP-RNAi/+	**0.22 ± 0.05**	**-0.75 ± 0.07**	**-0.43 ± 0.09**	**0.03 ± 0.09**
	******	*******	******	**n.s.**
FB-Gal4/+;GlyS-RNAi/+	**0.25 ± 0.06**	**-0.22 ± 0.09**	**-0.48 ± 0.12**	**-0.18 ± 0.18**
	******	*****	*****	**n.s.**
N (column top to bottom)	**6/6/7**	**9/8/8**	**9/8/6**	**8/8/6**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Larval fat body dissection and PAS staining reveal, that the genetic tools are indeed sufficient to visibly knock down gene expression regulating carbohydrate storage in the fat body (Figure 30A).

Next, appetitive olfactory conditioning with 2 M sucrose as reinforcer was performed. Here, neither upregulation of fat body glycogen nor downregulation of fat body glycogen resulted in significant changes to appetitive STM using 2 M sucrose as reinforcer (Figure 30B). The fat body appears to function primarily as a carbohydrate storage. Whether a complete depletion of fat body glycogen would result in appetitive STM changes, cannot be addressed with this assay. However, it can be said that *Drosophila* behaviour is not as sensitive to changes in fat body glycogen as to flight muscle glycogen.

Whole-body glycogen measurements of adult *Drosophila melanogaster* showed that fat body glycogen upregulation results in measurable changes to the overall carbohydrate storage (Figure 30C). However, downregulation of fat body glycogen did not alter whole-body glycogen levels, as measured with the ELISA reader and the Glucose Assay Kit. This could be because RNAi mediated knockdown in adult fat body is not sufficient to significantly reduce the levels. Utilising another fat body Gal4 line, such as *CG-Gal4* (Hennig et al., 2006) could provide an answer.

Next, the influence of fat body glycogen on appetite and hunger driven sucrose consumption was analysed over 3 h (Figure 30D) and 24 h, respectively (Figure 30E). Glycogen alterations of the fat body storage did not affect sucrose intake. Neither up- nor downregulation significantly altered consumption of 0.15 M sucrose. Consumption over 24 h, guided by appetite, was also not altered by up- or downregulation of fat body glycogen. All groups consumed similar amounts of the offered 0.15 M sucrose solution. Taken together, the fat body glycogen levels are not interfering with appetitive STM or sucrose intake and seems to not communicate the internal state from the fat body to the brain.

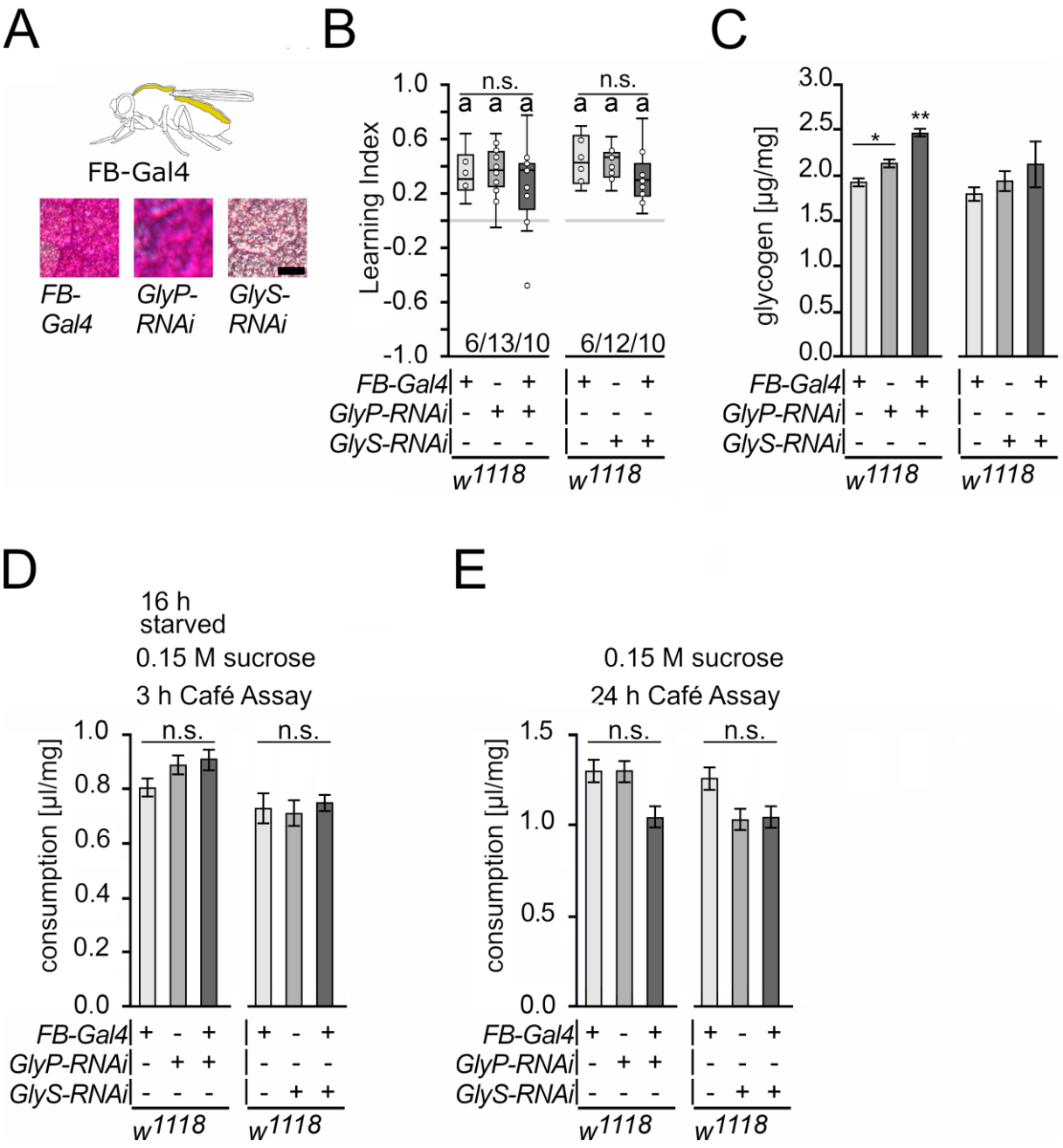

Figure 30: Fat body glycogen levels do not affect appetitive STM or sucrose intake

A, schematic overview of adult fat body and larval fat body PAS staining with RNAi mediated knockdown of *GlyP* and *GlyS.* Scale bar = 25 µm. **B**, appetitive STM with 2 M sucrose. Upregulation of glycogen in fat body does not influence appetitive memory formation. Furthermore, downregulation of fat body glycogen does not influence appetitive STM formation either. N = 6, 13, 10; 6, 12, 10. **C**, whole-body glycogen measurements with the Glucose Assay Kit. Upregulation of fat body glycogen significantly increased whole-body glycogen levels. However, downregulation of glycogen did not alter whole-body glycogen levels significantly. N = 3, 3, 3; 3, 3, 3. **D**, 3 h CAFE assay with RNAi mediated up- or downregulation of fat glycogen with 0.15 M sucrose. Hunger in 16 h starved animals does not influence carbohydrate consumption, when fat body glycogen is up- or downregulated. N = 29, 26, 23; 15, 30, 30. **E**, 24 h CAFE assay with RNAi mediated up- or downregulation of fat body glycogen with 0.15 M sucrose. Appetite is not altered by up- or downregulation of glycogen in the fat body. N = 13, 30, 24; 13, 30, 30. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Fat body and muscle glycogen regulate appetitive STM negatively

On the one hand, *Drosophila melanogaster* muscle glycogen levels are communicated to the brain as it results in changes of appetitive behaviour, such as increased STM upon decreased glycogen levels. On the other hand, glycogen in the fat body appears to be primarily a reserve for starvation periods, because neither appetitive STM nor sucrose intake was altered.

However, knowing that the *Drosophila* fat body functions in a similar way to the mammalian liver, the glucose monitor of the organism (Adeva-Andany et al., 2016) but divides the liver related functions between itself and the mid-gut (Hoshizaki, 2005) and that -at least- in larvae the fat body and lipogenesis functions as a safeguard for caloric overload to reduce lethality (Musselman et al., 2013; Yamada et al., 2018) it was interesting to further investigate the interaction between the fat body and muscles regarding a potential shared responsibility to regulate appetitive STM or food intake.

To investigate the role of flight muscle and fat body glycogen in regulation of appetitive memory formation, *UAS-GlyP-RNAi* and *UAS-GlyS-RNAi* was expressed under *FB-Gal4; mef2-Gal4* control and appetitive conditioning experiments were performed with 2 M sucrose reinforcement.

Beforehand, odorant acuity and sucrose sensing of the animals was checked to establish that the flies prefer the sucrose reward and avoid both odorants equally (Table 12). All groups show attraction towards 2 M sucrose reinforcer and avoid both odorants and show balanced behaviour when presented with both odorants simultaneously.

Table 12: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (FB;mef2)

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
FB-Gal4/+;mef2-Gal4/+	**0.19 ± 0.09**	**-0.39 ± 0.05**	**-0.43 ± 0.09**	**0.06 ± 0.08**
	*****	*******	******	**n.s.**
FB-Gal4/+;GlyP-RNAi/mef2-Gal4	**0.31 ± 0.06**	**-0.52 ± 0.06**	**-0.39 ± 0.06**	**0.03 ± 0.05**
	******	*******	*******	**n.s.**
FB-Gal4/+;GlyS-RNAi/mef2-Gal4	**0.22 ± 0.05**	**-0.71 ± 0.09**	**-0.73 ± 0.05**	**-0.07 ± 0.08**
	******	*******	*******	**n.s.**
N (column top to bottom)	**7/6/6**	**18/9/6**	**8/10/8**	**13/10/13**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Larval flight muscles and fat body were dissected and underwent PAS staining procedure to analyse glycogen content within the animal (Figure 31A). In larvae that expressed *UAS-GlyP-RNAi* under *FB-Gal4; mef2-Gal4* control, the pink colouration appears stronger, which was expected. Interestingly and surprisingly, larvae that expressed *UAS-GlyS-RNAi* under *FB-Gal4; mef2-Gal4* control, the pink colouration in the fat body was visibly reduced but the colouration in the muscles remained, which suggests that glycogen was primarily depleted from the fat body, supporting the hypothesis of the fat body as a glucose monitor for other organs.

Next, appetitive memory capability in flies with up- or downregulation in flight muscle and fat body was analysed (Figure 31B). Flies that expressed *UAS-GlyP-RNAi* under *FB-Gal4; mef2-Gal4* control showed significantly reduced appetitive memory scores in comparison to both genetic controls. Furthermore, flies that express *UAS-GlyS-RNAi* under *FB-Gal4; mef2-Gal4* control showed significantly increased memory after

appetitive olfactory conditioning. Thus, glycogen levels in both organs together regulate appetitive memory.

Last, whole-body glycogen was measured in flies with up- and downregulated glycogen levels in muscle and fat body (Figure 31C). Indeed, when glycogen is upregulated under *FB-Gal4; mef2-Gal4* control, whole-body glycogen is significantly increased. Additionally, when glycogen is downregulated by RNAi-mediated *GlyS* knockdown in *FB-Gal4; mef2-Gal4* targeted cells, flies show significantly decreased whole-body glycogen.

Taken together, flight muscle and fat body together regulate appetitive STM formation in an internal state dependent manner. Furthermore, the results indicate that the fat body in *Drosophila melanogaster* provides a similar monitoring role to the mammalian liver. Potentially providing energy to other organs without influencing appetitive STM or sucrose intake.

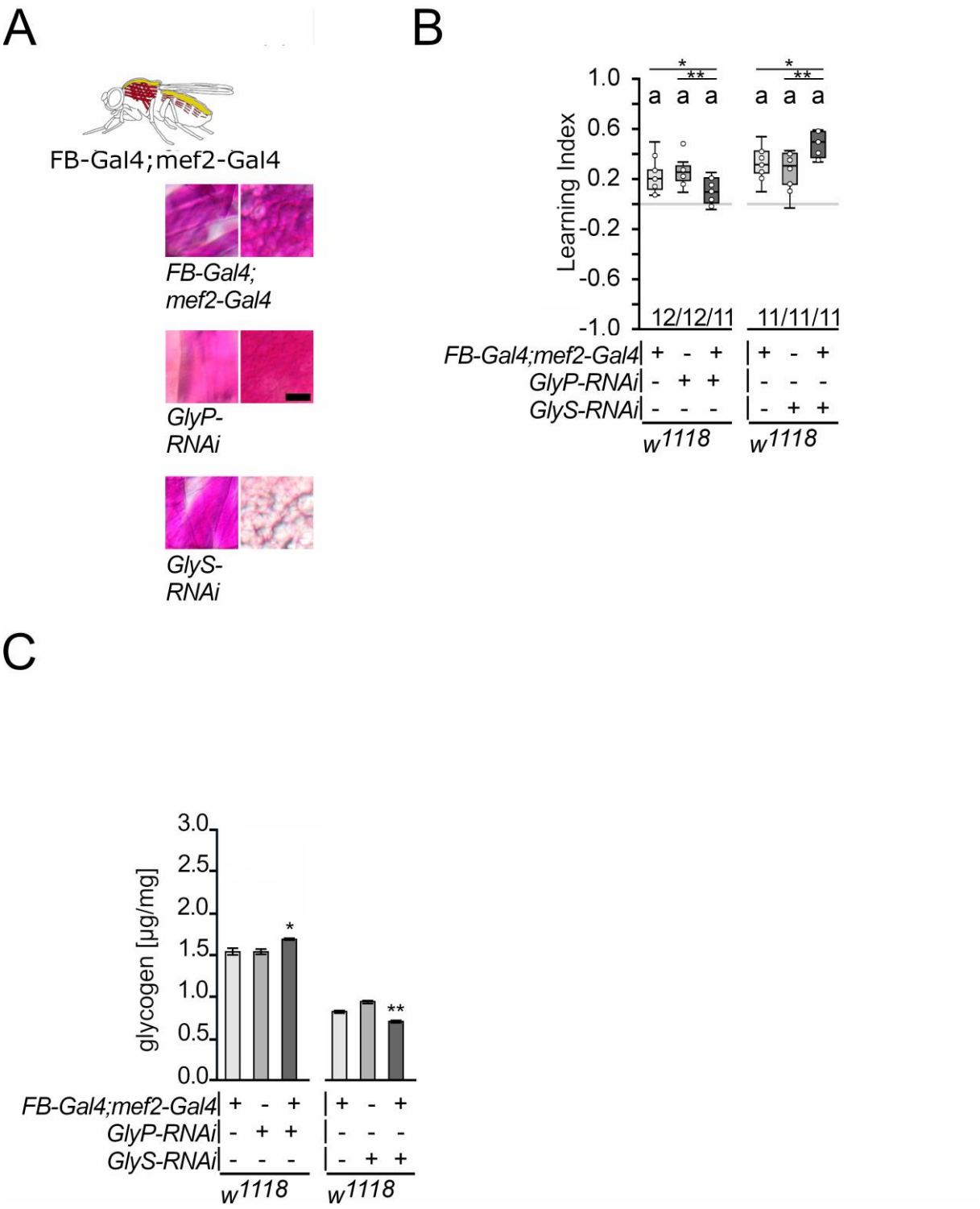

Figure 31: Simultaneous alteration of glycogen in flight muscle and fat body regulates appetitive STM negatively.

A, schematic overview of adult muscle and fat body as well as larval muscle and fat body PAS staining with RNAi mediated knockdown of *GlyP* and *GlyS.* The PAS staining shows (from top to bottom): genetic control. RNAi mediated knockdown of *GlyP* in muscle and fat body. RNAi mediated knockdown of *GlyS* in muscle and fat body. Scale bar = 25 µm. **B**, appetitive STM with 2 M sucrose reinforcement. Upregulation of glycogen in flight muscle and fat body simultaneously resulted in significantly decreased

memory scores in the experimental group. Downregulation of glycogen in flight muscle and fat body simultaneously significantly increased learning scores in flies. N = 12, 11, 11; 11, 11, 11. **C**, whole-body glycogen measurements with up- and downregulation in flight muscle and fat body simultaneously. Significantly increased whole-body glycogen in experimental flies with upregulated glycogen storage. Furthermore, decreased whole-body glycogen in experimental flies with downregulated glycogen storage. N = 3, 3, 3; 3, 3, 3. Difference from random choice was calculated with the one sample t-test. "a" above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Reduction of glycogen levels in muscle in *Tβh^{nM18}* did not improve appetitive memory defect

Tβh^{nM18} form appetitive STM in an internal state dependent manner. They form appetitive LTM under sated conditions. Upon starvation memory changes from LTM to ARM. RNAi-mediated *GlyS* knockdown significantly improved appetitive STM in flies with the *w^{1118}*.

To investigate, whether glycogen downregulation in flight muscles in the *Tβh^{nM18}* mutant background can induce internal state dependent 2-min memory, appetitive STM conditioning was performed with 2 M sucrose reinforcement. Sucrose attraction and odorant acuity were analysed as well (Table 13). All tested groups show attraction to sucrose and avoidance to the conditioned stimuli. Furthermore, flies show balanced behaviour towards simultaneous naïve presentation of both odorants.

Table 13: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (mef2) in *Tβh^{nM18}* mutant background

	Reinforcer attraction	**Odorant avoidance**		**Odorant balance**
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
Tβh^{nM18}/y; ;mef2-Gal4/+	**0.31 ± 0.05**	**-0.37 ± 0.10**	**-0.38 ± 0.08**	**-0.10 ± 0.06**
	******	******	******	**n.s.**
Tβh^{nM18}/y; ;GlyS-RNAi/+	**0.25 ± 0.06**	**-0.56 ± 0.05**	**-0.49 ± 0.06**	**-0.23 ± 0.17**
	*****	*******	*******	**n.s.**
Tβh^{nM18}/y; ;GlyS-RNAi/mef2-Gal4	**0.38 ± 0.09**	**-0.33 ± 0.06**	**-0.20 ± 0.05**	**-0.03 ± 0.02**
	******	******	*****	**n.s.**
N (column from top to bottom)	**8/8/9**	**6/8/7**	**7/9/6**	**7/9/9**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Larval body-wall muscle glycogen was dissected and underwent PAS staining procedure to analyse downregulation of glycogen storage in the *Tβh*^{nM18} mutants (Figure 32A). Interestingly, the pink colouration –indicating carbohydrate presence- was still present. This suggests that the genetic tools used might not provide enough power to deplete *Tβh*^{nM18} overall increased glycogen storage significantly.

However, knowing that even small amounts of depleted glycogen in flight muscles resulted in significant alteration of appetitive memory formation (Figure 29), the effects of depletion on appetitive memory formation was analysed in *Tβh*^{nM18} mutant background (Figure 32B).

Nevertheless, memory performance in experimental groups did not increase, which was unexpected. Only the genetic control was able to form appetitive 2-min memory and all groups in the *Tβh*^{nM18} genetic background did not show difference from random choice during test.

Next, 0.15 M sucrose consumption over 24 h was measured with the CAFE assay (Figure 32C). Here, all *Tβh*^{nM18} mutants consumed significantly less than the genetic control. All *Tβh*^{nM18} mutants consumed equal amounts of sucrose over 24 h.

To address the potential role of mushroom body expression of *Gal4* (Crittenden et al., 2018), flies that carried the *mb247-Gal80* transgene were crossed with mef2-Gal4 driver flies, and the transgene combination was crossed into the *Tβh*^{nM18} background. Indeed, blocking *Gal4* expression in the mushroom bodies while simultaneously knocking down *GlyS* expression resulted in significantly increased sucrose consumption, which was equal to transgenic control in the *w*^{1118} background control (Figure 32D). Thus, appetite is regulated by levels of glycogen in the fly muscles.

Taken together, the results show that flight muscle glycogen internal state alone does not affect appetitive memory in *Tβh*^{nM18} flies, but significantly improves sucrose consumption.

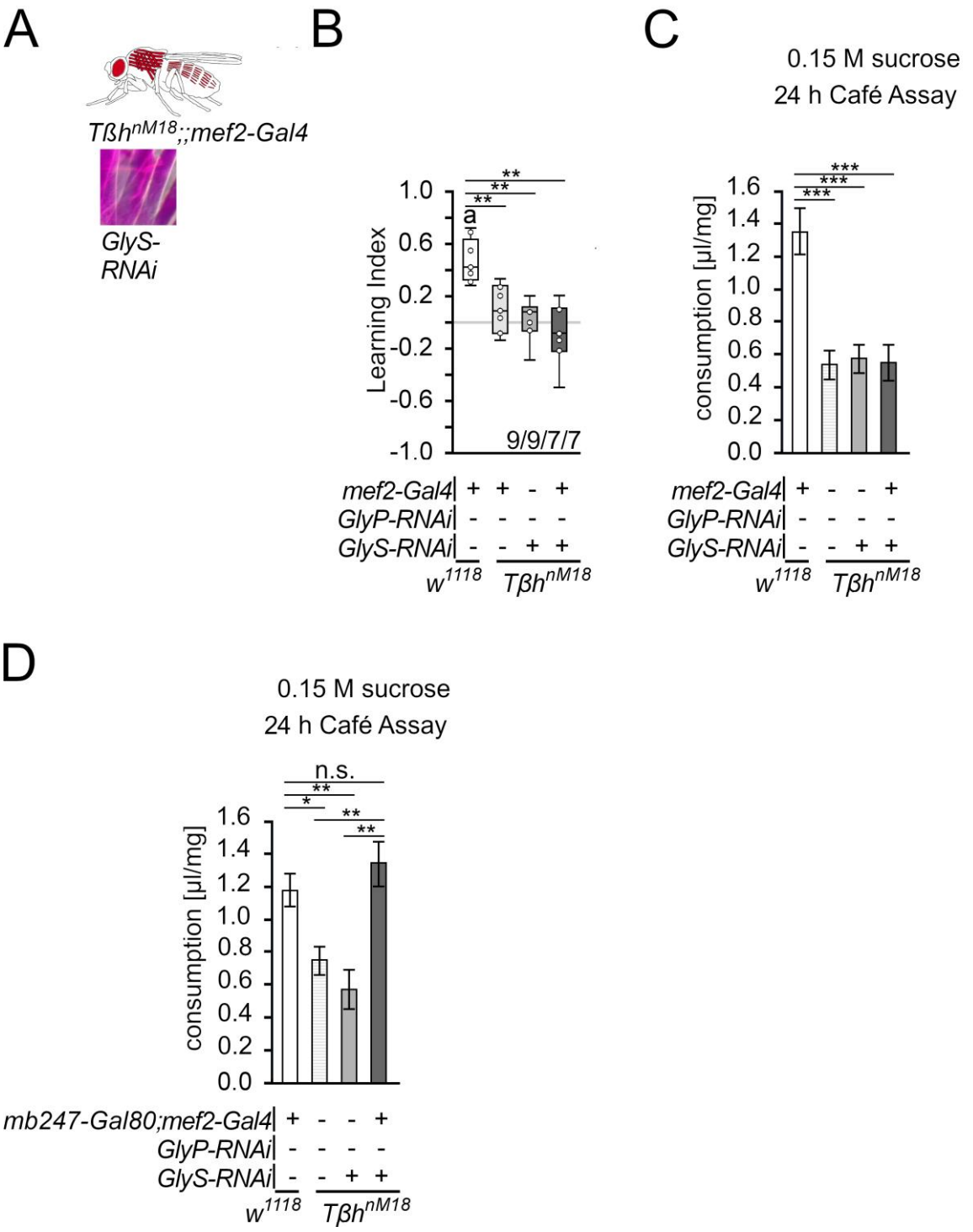

Figure 32: Flight muscle glycogen storage depletion alone does not rescue appetitive STM defects in *Tβh^{nM18}* flies.

A, schematic overview of adult flight muscle as well as larval body-wall muscle PAS staining with RNAi mediated knockdown of *GlyS* in the *Tβh^{nM18}* mutant background. **B**, appetitive STM with 2 M sucrose reinforcement. RNAi-mediated knockdown of *GlyS* in *mef2-Gal4* targeted cells in the *Tβh^{nM18}* mutant background did not significantly improve appetitive STM. N = 9, 9, 7, 7. **C**, 24 h CAFE assay with 0.15 M sucrose solution. Decreased glycogen in flight muscles in the *Tβh^{nM18}* mutant background did not increase consumption over *Tβh^{nM18}* genetic control levels. N = 20, 14, 15, 15. **D**, 24 h CAFE assay with

0.15 M sucrose solution. Blocking Gal4 expression in the mushroom bodies with mb247-Gal80 driver, significantly increased sucrose consumption in *Tβh^{nm18}* background flies. N = 19, 15, 13, 15. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Tβh^{nM18} mutants do not show significantly increased appetitive STM or food intake after fat body glycogen depletion

To investigate, whether the fat body glycogen level could influence appetitive STM or sucrose intake in *Tβh^{nM18}* mutant flies, appetitive STM conditioning and CAFE assays were performed. In *w^{1118}* flies, appetitive behaviour was not altered by artificial depletion of fat body glycogen storage (Figure 30). Therefore, the assumption was, that fat body glycogen alone would also not significantly affect appetitive behaviour in *Tβh^{nM18}* flies.

Odorant acuity and attraction towards the appetitive reinforcer was measured prior to learning and memory experiments (Table 14). All groups significantly preferred the offered reinforcer and avoided the used odorants. In a simultaneously offered naïve choice situation both odorants were equally attractive or aversive.

Table 14: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (FB) in *Tβh^{nM18}* mutant background

	Reinforcer attraction	**Odorant avoidance**		**Odorant balance**
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
Tβh^{nM18}/y; FB-Gal4/+	**0.23 ± 0.07**	**-0.62 ± 0.06**	**-0.60 ± 0.08**	**-0.20 ± 0.13**
	******	*******	*******	**n.s.**
Tβh^{nM18}/y; GlyS-RNAi/FB-Gal4	**0.31 ± 0.07**	**-0.58 ± 0.08**	**-0.54 ± 0.11**	**-0.12 ± 0.25**
	******	*******	******	**n.s.**
N (column top to bottom)	**8/7**	**8/6**	**9/7**	**9/7**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001. N = 8, 8, 9, 9; 7, 6, 7, 7.

Fat body glycogen in *Tβh*^{nM18} larvae with RNAi-mediated *GlyS* knockdown was visible through pink colouration (Figure 33A). However, many transparent spots can be observed within each cell, indicating reduction of glycogen in the larval fat body.

Next, appetitive STM was trained with 2 M sucrose reinforcement. The genetic control in the *w*^{1118} background showed significant and robust 2-min memory after one-cycle appetitive conditioning (Figure 33B). As expected, all groups in the *Tβh*^{nM18} genetic background did not show appetitive 2-min memory after training. Therefore, it can be concluded that the fat body glycogen storage is not regulating internal-state dependent appetitive memory formation in *Tβh*^{nM18} flies at least not in the range, in which the genetic tools are able to decrease glycogen levels in the fat body.

Next, sucrose consumption was measured over 3 h (Figure 33C) and 24 h (Figure 33D) in *Tβh*^{nM18} mutant flies with RNAi-mediated downregulation of *GlyS* in the fat body. However, hunger and appetite mediated consumption was not altered in flies that were offered 0.15 M sucrose solution. All groups consumed equal amounts, which was surprising, as a lower consumption of the *Tβh*^{nM18} mutants would have been expected. Alas, a clear conclusion about fat body glycogen guided regulation of sucrose consumption behaviour cannot be drawn from these results. Potential reasons for the unexpected behaviour will be discussed further below, in the Discussion chapter.

Taken together, the results suggest that fat body glycogen functions most likely as a storage for glycogen that might be used as back up for other organs in time of need.

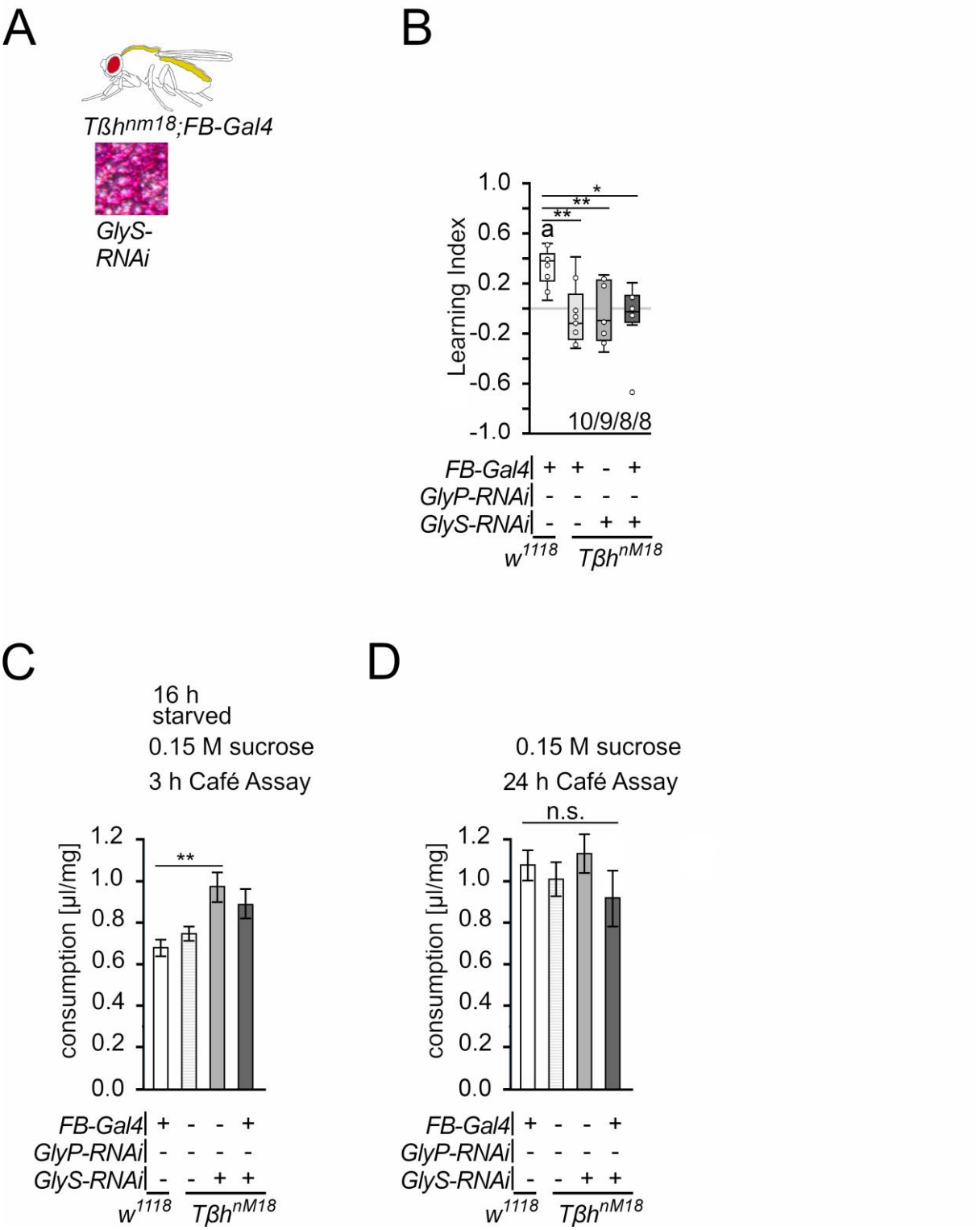

Figure 33: Fat boy glycogen storage depletion alone does not rescue appetitive STM defects in *Tβh^{nM18}* flies.

A, schematic overview of adult fat body as well as larval fat body PAS staining with RNAi mediated knockdown of *GlyS* in the *Tβh^{nM18}* mutant background. **B**, appetitive STM with 2 M sucrose reinforcement. RNAi-mediated knockdown of *GlyS* in *FB-Gal4* targeted cells in the *Tβh^{nM18}* mutant background did not significantly improve appetitive STM. N = 10, 9, 8, 8. **C**, 3 h CAFE assay with 0.15

M sucrose solution. Decreased glycogen in fat body in the *Tβh*^{nM18} mutant background did not increase consumption over *Tβh*^{nM18} genetic control levels. N = 19, 14, 12, 7. **D**, 24 h CAFE assay with 0.15 M sucrose solution. Decreased glycogen in fat body in the *Tβh*^{nM18} mutant background did not increase consumption over *Tβh*^{nM18} genetic control levels. N = 21, 29, 18, 11. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Muscle and fat body glycogen regulate appetitive STM formation in Tβh^{nM18} mutants

Starvation and alteration of the internal state regulates appetitive learning and memory in *Drosophila melanogaster* (Figure 24, 27 and 29). However, in *Tβh*^{nM18} mutants appetitive STM with sucrose reward was only observable in virgin female flies but not in male flies. Therefore, the results suggest that male *Tβh*^{nM18} are indeed deficient for appetitive STM formation with sucrose reinforcement. Furthermore, artificial alteration of glycogen storage utilising the Gal4/UAS-system in either muscle or fat body appears to have no measurable effect on appetitive memory formation in *Tβh*^{nM18} mutant.

To ascertain the assumption further, that *Tβh*^{nM18} flies are deficient for internal state regulated appetitive STM with sucrose reinforcement, glycogen storage was decreased in muscle and fat body simultaneously with RNAi mediated knockdown of *GlyS* (Figure 34).

At the start, the odorant acuity and sucrose attraction were measured to ensure equal aversion of odorants and attraction towards the offered reinforcer in all genotypes that were previously not used in other experiments (Table 15). In all groups, odorant avoidance and balance were established. Furthermore, the reinforcer 2 M sucrose was attractive to naïve flies.

Table 15: Odorant acuity and control experiments in *GlyP* and *GlyS* knockdown flies (FB;mef2) in *Tβh^{nM18}* mutant background

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
Tβh^{nM18}/y; FB-Gal4/+; mef2-Gal4/+	**0.23 ± 0.05**	**-0.20 ± 0.03**	**-0.35 ± 0.07**	**-0.28 ± 0.21**
	******	*******	******	**n.s.**
Tβh^{nM18}/y;FB-Gal4;GlyS-RNAi/mef2-Gal4	**0.26 ± 0.07**	**-0.19 ± 0.08**	**-030 ± 0.05**	**-0.08 ± 0.08**
	******	*****	******	**n.s.**
N (column top to bottom)	**6/8**	**6/7**	**6/6**	**8/9**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Larval body-wall tissue and larval fat body was dissected, and PAS staining was performed (Figure 34A). In both, the body-wall and fat body, glycogen storage was still observable, as indicated by the slight pink colouration. However, in comparison to *Tβh^{nM18}* larval body-wall and fat body, the colour is less intense, which suggests that the genetic tools used were strong enough to reduce increased glycogen levels in *Tβh^{nM18}* mutants.

Notably, the colouration was less intense in the fat body than the body-wall muscles, which supports the idea that the fat body is first to be depleted before all other organs and fits with observation in literature, where the fat body glycogen reserves are exhausted first (Wigglesworth, 1949).

Next, appetitive olfactory conditioning with 2 M sucrose reinforcement was performed (Figure 34B). Here, the controls show appetitive STM after one-cycle of STM conditioning. The two groups of the *Tβh^{nM18}* mutant show 2-min memory scores that are not significantly different from random choice and are significantly different to the *w^{1118}* background control group. Furthermore, the experimental group shows no significant difference to the *w^{1118}* background control group and shows appetitive memory that is significantly different from random choice.

Therefore, monitoring the glycogen storage in the flight muscles and fat body of *Tβh*^{nM18} is necessary and sufficient to elicit appetitive STM in *Tβh*^{nM18} mutants.

To determine, if the observed memory in artificially glycogen depleted *Tβh*^{nM18} mutants is STM and not rapidly consolidated ARM or LTM, the glycogen levels were measured in all experimental groups that were tested in the *Tβh*^{nM18} mutant background (Figure 34C). Indeed, all groups of *Tβh*^{nM18} mutants show significantly increased whole-body glycogen in comparison to the *w*^{1118} background control. Interestingly, all experimental groups show no significant whole-body glycogen depletion overall, suggesting that behavioural regulation through signalling of internal state is a very fine-tuned mechanism that is not in need of drastic changes of glycogen levels.

Seeing that only significant depletion of glycogen through starvation induced rapidly consolidated ARM in *Tβh*^{nM18} mutants, it can be concluded that *Tβh*^{nM18} male flies are indeed capable of forming appetitive, internal-state dependent STM.

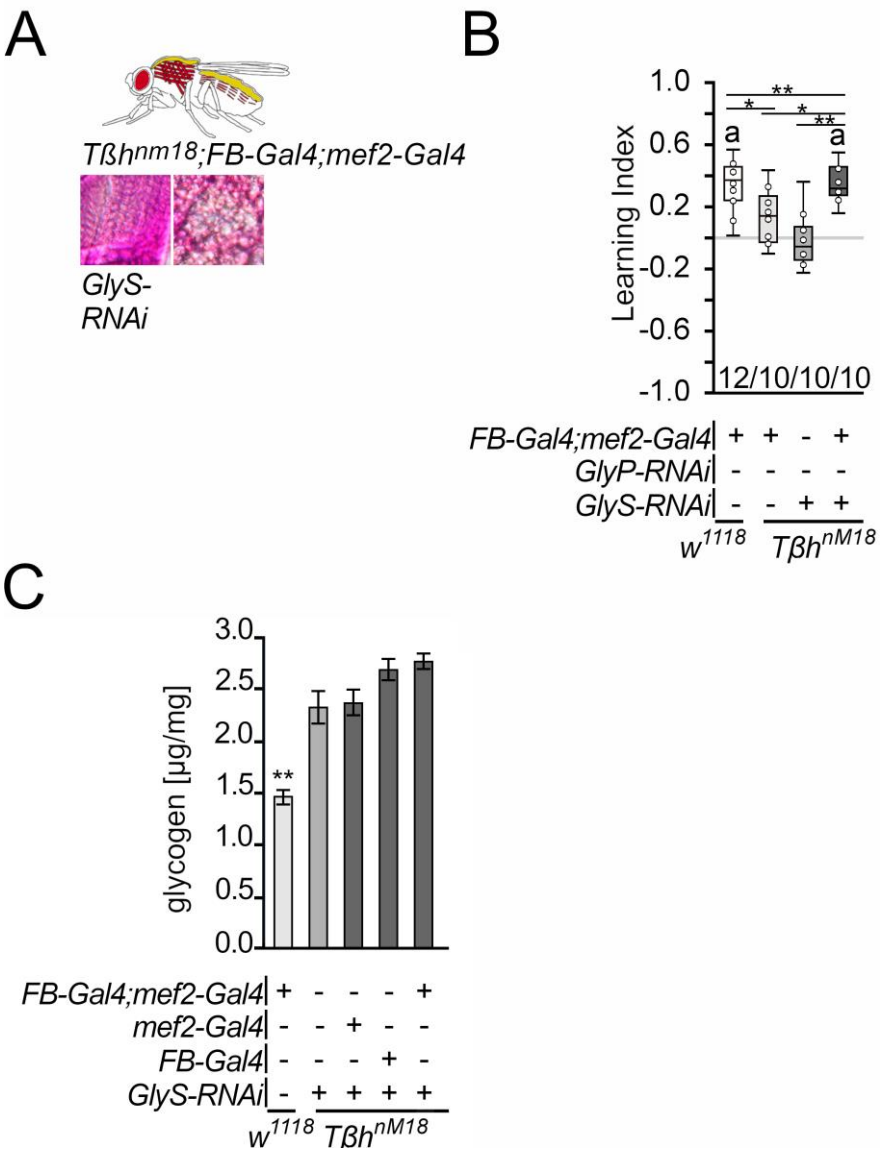

Figure 34: Muscle and fat body glycogen storage together regulate appetitive STM in *TβH^{nM18}* flies.

A, schematic overview of adult muscle and fat body as well as larval body-wall and fat body PAS staining with RNAi mediated knockdown of *GlyS* in the *TβH^{nM18}* mutant background. **B**, appetitive STM with 2 M sucrose reinforcement. Downregulation of glycogen in muscle and fat body simultaneously induces appetitive STM after conditioning in *TβH^{nM18}* flies. N = 12, 10, 10, 10. **C**, whole-body glycogen measurement in all experimental groups in the *TβH^{nM18}* mutants. Glycogen levels in all *TβH^{nM18}* mutant groups are significantly higher than in the *w^{1118}* control background flies. N = 3, 3, 3, 3, 3. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Internal-state dependent regulation of appetitive STM or food intake is mediated through starvation. One major player in communication of the internal state in the organism is insulin in vertebrates and insulin-like signalling in invertebrates (Ikeya et al., 2002; Kapan et al., 2012; Mattila and Hietakangas, 2017; Nässel et al., 2015; Saltiel and Kahn, 2001).

To investigate the role of insulin-like signalling as regulator of appetitive behaviour through OA neurons, appetitive STM conditioning and CAFE assays were performed with OE of either *InR^{dn}* or *InR^{ca}* in *Tdc2-Gal4* targeted OA neurons, to artificially alter internal-state signalling (Figure 35).

Odorant acuity and sucrose attraction was measured in all groups participating in olfactory conditioning to establish that neither odorant is more aversive than the other and that sucrose is a rewarding reinforcer (Table 16). Indeed, all groups show aversion to 3-Oct (1:100) and MCH (1:80) and are attracted to 2 M sucrose.

Table 16: Odorant acuity and control experiments in fruit fly line *Tdc2-Gal4* with expressed *UAS-InR^{dn}* or *UAS-InR^{ca}*.

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
Tdc2-Gal4/+	**0.23 ± 0.06**	**-0.23 ± 0.09**	**-0.23 ± 0.08**	**0.02 ± 0.09**
	******	*****	*****	**n.s.**
UAS-InR^{dn}/+	**0.20 ± 0.05**	**-0.29 ± 0.08**	**-0.25 ± 0.04**	**0.02 ± 0.06**
	******	*****	******	**n.s.**
UAS-InR^{ca}/+	**0.21 ± 0.05**	**-0.64 ± 0.09**	**-0.32 ± 0.08**	**-0.08 ± 0.05**
	******	******	******	**n.s.**
Tdc2-Gal4/+;UAS-InR^{dn}/+	**0.18 ± 0.05**	**-0.27 ± 0.08**	**-0.32 ± 0.07**	**0.05 ± 0.06**
	******	*****	******	**n.s.**
Tdc2-Gal4/UAS-InR^{ca}	**0.24 ± 0.05**	**-0.26 ± 0.09**	**-0.30 ± 0.10**	**-0.04 ± 0.19**
	******	*****	*****	**n.s.**
N (column top to bottom)	**7/13/7/8/8**	**11/6/6/6/12**	**11/6/9/10/8**	**13/13/13/10/6**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

First, appetitive STM was analysed by STM training with 2 M sucrose as reinforcer (Figure 35A). Both genetic controls form significant and stable appetitive STM. Furthermore, the experimental group with overexpression of *UAS-InR*^{dn}, shows equally significant and stable appetitive STM. This was unexpected, because blocking of insulin-like signalling was expected to result in increase of appetitive memory, due to neurons being unable to sense the internal-state.

Next, 3 h and 24 h CAFE assays were performed, to analyse how blocking insulin-like signalling would affect hunger and appetite driven sucrose intake. Blocking InR signalling onto *Tdc2-Gal4* targeted OA neurons significantly increased hunger-mediated sucrose consumption (Figure 35B). Furthermore, appetite-driven consumption is also regulated by InR signalling onto OA neurons (Figure 35C). Thus, insulin-like signalling onto OA neurons is regulating sucrose intake but not appetitive memory.

Next, appetitive STM was analysed by STM training with 2 M sucrose reinforcement with constative active *InR* variant OE in *Tdc2-Gal4* positive OA neurons (Figure 35D) to investigate whether constant insulin-like signalling on OA neurons regulates appetitive STM. Here, both genetic control groups formed appetitive STM that was significantly different from random choice. The experimental group also formed appetitive STM indifferent to both control groups.

Neither hunger-driven (Figure 35E) nor appetite-driven carbohydrate consumption (Figure 35F) was altered when InR signalling was constative active, simulating sated state mediated by *Tdc2-Gal4* targeted OA neurons.

Thus, OA neurons appear to be not responsible for satiety state communication to the brain.

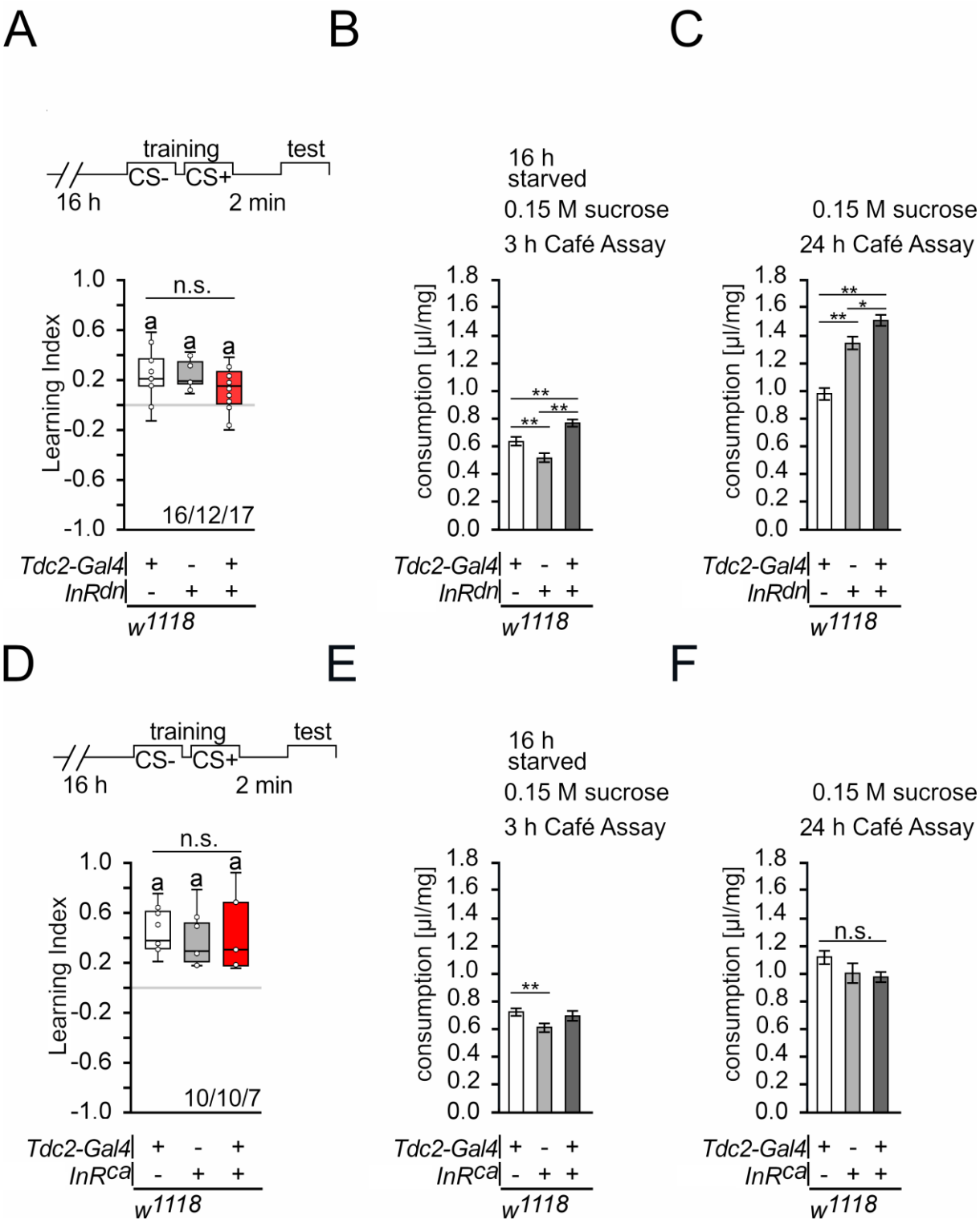

Figure 35: Insulin-like signalling onto *Tdc2-Gal4* targeted OA neurons regulates hunger and appetite driven consumption behaviour in *Drosophila melanogaster*.

A, appetitive STM with 2 M sucrose as reinforcer. Insulin-like signalling block through dominant negative *InR* OE in *Tdc2-Gal4* targeted neurons does not alter appetitive STM in *Drosophila melanogaster*. N = 16, 12, 17. **B**, 3 h CAFE assay with 0.15 M sucrose solution. Hunger driven consumption is negatively regulated by insulin-like signalling onto OA neurons. N = 30, 29, 30. **C**, 24 h CAFE assay with 0.15 M

sucrose solution. Appetite driven consumption behaviour is negatively regulated by insulin-like signalling onto OA neurons. N = 40, 40, 40.
C, appetitive STM with 2 M sucrose reinforcement. Insulin-like signalling through constative active *InR* OE in *Tdc2-Gal4* targeted neurons does not alter appetitive STM formation in *Drosophila melanogaster.* N = 10, 10, 7. **E**, 3 h CAFE assay with 015 M sucrose solution. Hunger driven consumption is not altered upon insulin-like signalling through OA neurons. N = 30, 24, 25. **F**, 24 h CAFE assay with 0.15 M sucrose solution. Appetite driven consumption behaviour is not altered through insulin-like signalling onto OA neurons. N = 30, 26, 29. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Next, to investigate the role of octopaminergic neurons in regulation of appetitive learning behaviour and to determine further whether satiety is indeed not regulated by OA positive *Tdc2-Gal4* neurons, appetitive STM conditioning with 2 M sucrose was performed with feeding of either water or 2 M sucrose for 15-min prior to conditioning (Figure 36).
H_2O fed flies show unaltered significant appetitive memory, while 2 M sucrose fed flies fail to show any appetitive STM.
Taken together, starvation-state, induced by block of InR signalling onto OA neurons, regulates sucrose consumption but not appetitive STM. Satiety, by constant activation of InR signalling onto OA neurons or feeding on carbohydrates prior to experiments however is not communicated via octopamine, seeing that in neither experiment appetitive behaviour was altered by OE of *UAS-InR*^{ca} and appetitive STM formation was blocked after 2 M sucrose consumption.

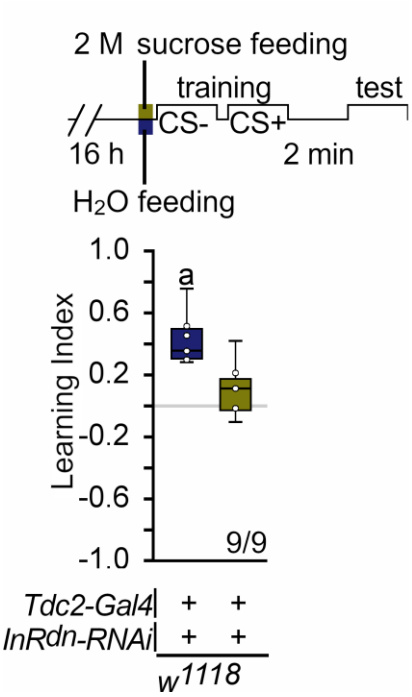

Figure 36: *Tdc2-Gal4* positive OA neurons are not solely responsible for communication of carbohydrate ingestion to the brain to alter behavioural output.

Appetitive STM training with 2 M sucrose reinforcement with prior 15-min feeding of H_2O or 2 M sucrose. Consumption of carbohydrates prior to appetitive conditioning blocks formation of appetitive memory in male flies with InR signalling onto *Tdc2-Gal4* positive OA neurons. N = 9, 9. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

OA neurons mediate internal-state dependent adaptations of appetitive behaviour

Blocking InR signalling onto neurons results in those neurons being unable to bind insulin-like peptides to communicate ingestion of carbohydrates to downstream targets and activate downstream cascades. OA neurons appear to be redundant in communication of internal-state information to regulate appetitive memory formation through insulin-like signalling. Due to the fact, that for an appetitive olfactory conditioning procedure, flies are starved for at least 16 h, the whole organism might be signalling “starvation”, which would result in masking the influence of OA neurons onto regulation of appetitive memory formation.

Still, it was interesting to see whether OA neurons might play a more important role in regulation of appetitive STM in *Tβh^{nM18}* mutants.

To analyse the hypothesis that *Tdc2-Gal4* targeted OA neurons are important for appetitive STM in *Tβh^{nM18}*, olfactory conditioning was performed with 2 M sucrose reinforcement (Figure 37). Furthermore, sucrose intake was also analysed in *Tβh^{nM18}* mutant background flies with blocked insulin-like signalling.

First, the odorant acuity and the sucrose attraction were analysed in all participating groups (Table 17). All groups showed attraction towards sucrose and avoided all used odorants and -more importantly- showed balanced behaviour towards both odorants, when they were presented simultaneously under naïve conditions.

Table 17: Odorant acuity and control experiments in fruit fly line *Tβh^{nM18}; Tdc2-Gal4* with expressed *UAS-InR^{dn}*.

	Reinforcer attraction	Odorant avoidance		Odorant balance
Genotype	**2 M sucrose approach**	**3-Oct (1:100) avoidance**	**MCH (1:80) avoidance**	**3-Oct (1:100) v MCH (1:80)**
Tβh^{nM18}/y; Tdc2-Gal4/+	**0.43 ± 0.08**	**-0.69 ± 0.05**	**-0.43 ± 0.08**	**-0.02 ± 0.04**
	*******	*******	******	**n.s.**
Tβh^{nM18}/y; UAS-InR^{dn}/+	**0.29 ± 0.10**	**-0.26 ± 0.11**	**-0.47 ± 0.09**	**-0.12 ± 0.14**
	*****	*****	*******	**n.s.**
Tβh^{nM18}/y; Tdc2-Gal4/UAS-InR^{dn}	**0.25 ± 0.08**	**-0.70 ± 0.05**	**-0.41 ± 0.09**	**-0.14 ± 0.11**
	*****	*******	******	**n.s.**
N (column top to bottom)	**8/7/6**	**7/9/11**	**8/10/7**	**11/10/9**

Mean ± s.e.m. is shown for the balance tests. Significant difference from random choice was established with the one-sample t-test. n.s. = p >0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

In appetitive conditioning experiments with 2 M sucrose as reinforcer, the genetic control was able to form appetitive STM after one-cycle training (Figure 37A). Both *Tβh^{nM18}* mutants showed no memory 2 min after conditioning with sucrose reward. Interestingly, the experimental group, with blocked InR signalling onto OA positive *Tdc2-Gal4* neurons, showed significantly improved appetitive 2-min memory that was on equal levels with the *w^{1118}* background control.

To analyse whether InR block induced appetitive STM and blocked the LTM formation capabilities of *Tβh^{nM18}* mutants, appetitive conditioning with 2 M sucrose was performed with cold shock 2 h after training. The test was performed 3 h after training (Figure 37B).

Interestingly, the genetic control in the *w^{1118}* background showed memory 3 h after one-cycle STM conditioning, which consisted of an ARM and an ASM part, seeing that cold shock did significantly reduce but did not completely erase appetitive memory. This suggests that the presence of the Gal4 transgene might affect memory stability. The *Tβh^{nM18}* mutant background controls also show appetitive memory 3 h after training, which was not abolished by cold shock. This agrees with prior results (Figure 25).

Most surprising was, that the experimental group also formed memory 3 h after training that was not abolished by cold shock. This suggests, that appetitive STM was formed independently of appetitive LTM, seeing that at 16 h starvation *Tβh^{nM18}* flies normally do not show appetitive 2-min memory but upon *InR^{dn}* OE do (Figure 37A). Therefore, InR signalling through OA neurons is necessary for appetitive STM regulation but is not affecting appetitive LTM formation, because LTM is not altered by *InR^{dn}* expression.

Next, the importance of InR signalling onto OA neurons in *Tβh^{nM18}* mutant background on sucrose consumption was analysed. For this, 3 h and 24 h CAFE assays were performed, to determine InR signalling importance for hunger-driven (Figure 37C) and appetite-driven consumption behaviour (Figure 37D). While hunger-driven behaviour is independent of InR signalling onto *Tdc2-Gal4* positive neurons, appetite-driven consumption behaviour is negatively regulated by it. Blocking InR-signalling in OA neurons in the *Tβh^{nM18}* background resulted in significantly increased consumption of 0.15 M sucrose solution in comparison to the other *Tβh^{nM18}* control groups. Consumption was like the *w^{1118}* genetic background control.

Taken together, InR signalling onto OA neurons regulates appetitive STM formation and sucrose intake in appetite-driven contexts. However, the effects of OA neuronal signalling are only visible in organisms with increased carbohydrate levels, otherwise other “starved” neurons appear to be upstream and thus more important for appetitive learning behaviour.

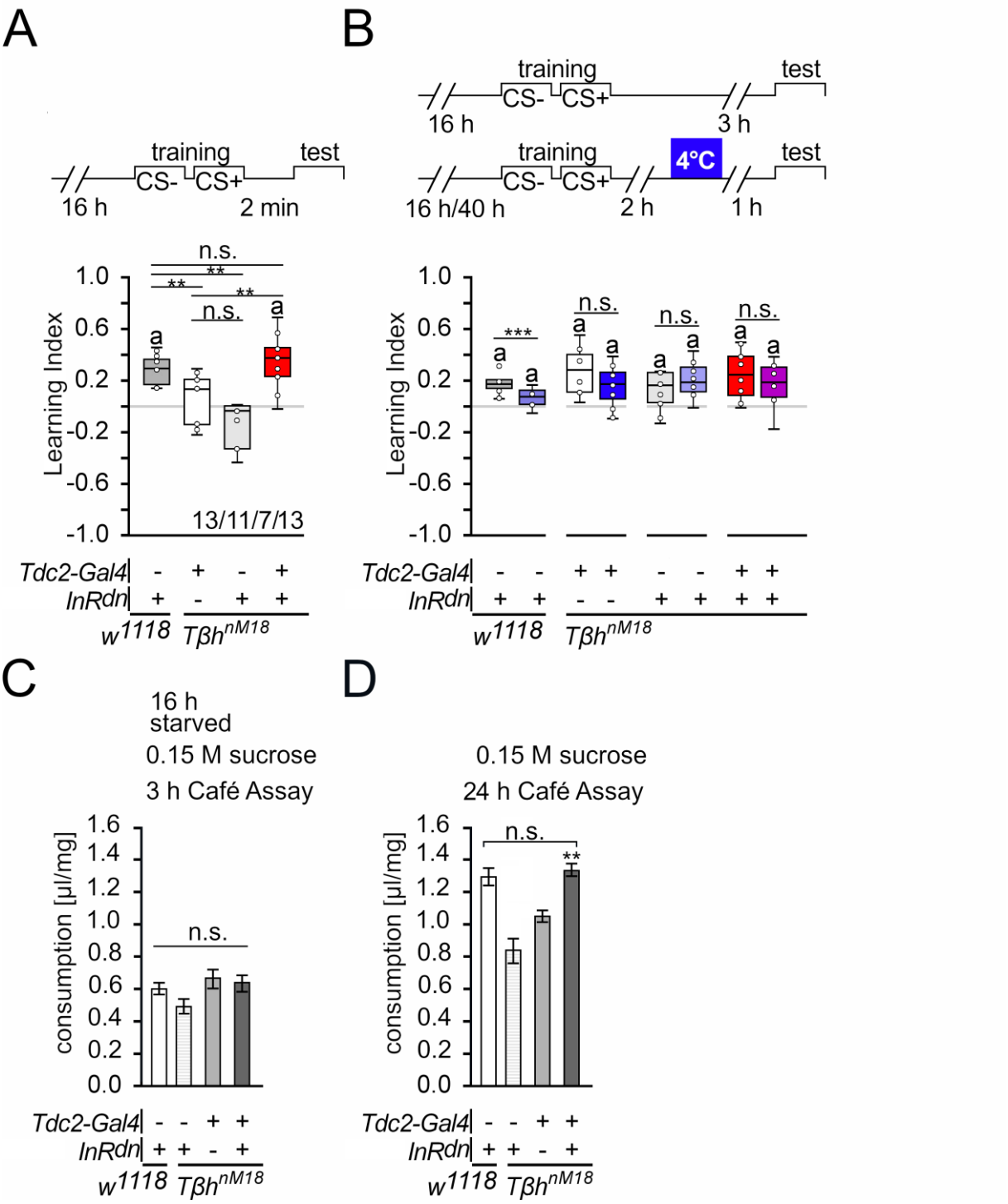

Figure 37: Insulin-like signalling onto OA neurons regulates appetitive memory formation and appetite-driven carbohydrate consumption in *TβhnM18* mutant background flies.

A, appetitive STM with 2 M sucrose reinforcement. Block of InR signalling on OA neurons significantly improved appetitive STM formation in *TβhnM18* flies. N = 13/11/7/13. **B**, appetitive conditioning with 2 M sucrose reinforcement and cold shock 2 h after training. Memory of *TβhnM18; Tdc2-Gal4/UAS-InRdn* flies cannot be erased by cold-shock, indicating that appetitive LTM is regulated independently of insulin-like signalling. N = 18, 18; 18, 17; 16, 16; 17, 16. **C**, 3 h CAFE assay with 0.15 M sucrose. Hunger-driven consumption in *TβhnM18* mutants is independent of InR signalling onto OA neurons. N = 30, 19, 12, 15.

D, 24 h CAFE assay with 0.15 M sucrose. Appetite-driven consumption behaviour in *Tβh*^{nM18} is negatively regulated by InR signalling. N = 26, 20, 28, 20. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

OA is a negative regulator of appetitive STM and LTM

Lack of OA in *Tβh*^{nM18} mutants results in appetitive LTM formation exclusively, independent of internal state. Increased starvation, artificial alteration of *GlyS* expression or block of InR signalling can circumvent the appetitive STM defect in *Tβh*^{nM18} mutants. OA is known to regulate decision making regarding preferences in a choice situation (Claßen and Scholz, 2018). Therefore, it was interesting to investigate the potential role of OA as behavioural switch in learning behaviour as well.

To ascertain this assumption, pharmacological feeding experiments were performed, coupled with appetitive conditioning with 2 M sucrose reinforcement (Figure 38). When 3 mM OA, mixed with water, is fed for 16 h during the starvation period prior to conditioning experiments, memory in *w*^{1118} is not affected (Figure 38A). No significant differences between H_2O fed and 3 mM OA fed flies were observable.

However, when 3 mM OA is fed for 30 minutes prior to conditioning, *w*^{1118} can no longer form appetitive STM with 2 M sucrose reinforcement (Figure 38B). Memory is no longer different from random choice and significantly decreased in comparison to H_2O fed flies. These results suggest that OA signalling blocks appetitive STM formation.

Next, 3 mM Epinastine, an OA receptor antagonist that competes with OA for the receptor (Roeder et al., 1998), was fed for 1 h prior to test to *w*^{1118} that were trained in one-cycle STM conditioning and tested 6 h after training (Figure 38C). This way OA signalling was blocked.

While H_2O fed flies do not retain appetitive memory for 6 h, Epinastine fed flies indeed show significant appetitive memory. Thus, blocking OA signalling leads to the formation of LTM.

Next, *Tβh*^{nM18} mutants were fed with 3 mM OA between training and test. They were trained in a one-cycle STM training and tested 6 h after training (Figure 38D). Surprisingly, *Tβh*^{nM18} flies that were fed with OA did no longer form appetitive LTM, seeing that the memory scores were indifferent from random choice.

Taken together, OA is responsible for the block of appetitive LTM formation as well as appetitive STM formation. Thus, suggesting that OA functions as a gatekeeper for memory to reduce “nonsense” learning and coincidence encounters.

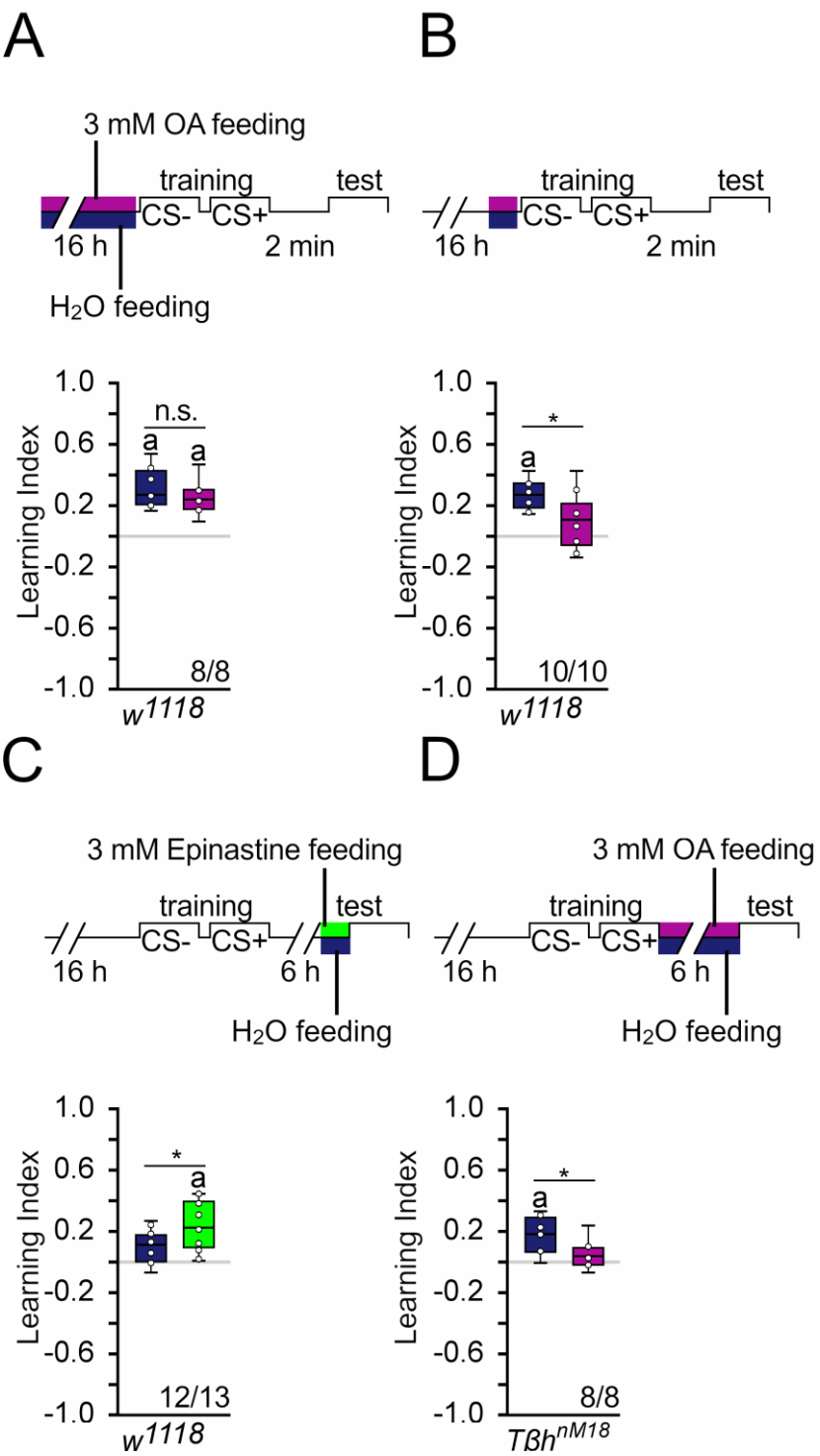

Figure 38: OA signalling functions as a memory formation switch, shifting memory from LTM to STM

A, appetitive STM with 2 M sucrose reinforcement. Constant 3 mM OA feeding during starvation does not affect appetitive memory formation in w^{1118}. N = 8, 8. **B**, appetitive STM with 2 M sucrose reinforcement. A short 30-minute period of 3 mM OA feeding blocks appetitive STM formation in w^{1118}. N = 10, 10. **C,** appetitive STM with 2 M sucrose reinforcement. 3 mM Epinastine feeding 1 h prior to test shifts appetitive memory towards LTM. N = 12, 13. **D**, appetitive STM with 2 M sucrose reinforcement. Feeding of 3 mM OA after training results in no appetitive LTM in $T\beta h^{nM18}$ 6 h after training. N = 8, 8. Difference from random choice was calculated with the one sample t-test. “a” above a box plot indicates p-values <0.05. Difference between more than two groups were calculated with a one-way ANOVA with post-hoc Bonferroni Holm correction. n.s. = p-value: >0.05; * = p-value <0.05; ** = p-value <0.01; *** = p-value <0.001.

Discussion

The role of ethanol as a reinforcer in olfactory associative conditioning in the *Drosophila melanogaster* larvae

Ethanol is attractive in a concentration dependent manner

Drosophila melanogaster larvae are attracted to most odorants (Fishilevich et al., 2005; Hoare et al., 2011; Kreher et al., 2008) which is crucial for the animal because odours in nature are often signifiers for food sources (Engel and Tressl, 1983; Jirovetz et al., 2003). Most of the natural odorants activate larval OR (Kreher et al., 2005). The same is true for olfactory attraction towards ethanol as an odorant (Figure 13). Ethanol in the environment is present in rotting fruit due to fermentation processes and is thus a cue for food (Geer et al., 1985; Giang et al., 2017; Lynch et al., 2017; Parsons, 1980; Rao and Stokes, 1953). Adult *Drosophila* lay their eggs on rotting fruits and larvae develop in an ethanol enriched environment, where fermentation leads to increasing ethanol concentrations over time (Geer et al., 1985; Kacsoh et al., 2013; McKenzie and Parsons, 1972; Milan et al., 2012; Richmond and Gerking, 1978; Schumann et al., 2021). Therefore, an innate attraction is not surprising. Indeed, in olfactory attraction assays performed with larvae, ethanol was shown to be attractive to the animal when offered on two equidistant points of a petri dish as an odorant (Khurana and Siddiqi, 2013). Ethanol attraction increased with increased concentration and reached a maximum with undiluted ethanol.

However, in this thesis larvae show a concentration dependent decline in attraction towards ethanol as an odorant that is shaped like a Gaussian bell curve with genotype specific preference maxima.

While *CantonS* appears to show attraction towards higher concentrations of ethanol (8 %), w^{1118} shows a preference shift towards lower concentrations (5 %). A concentration dependent behavioural decline to odorants, as well as other alcohols, was already reported for other volatile compounds (Khurana and Siddiqi, 2013), but interestingly, not for ethanol. This could be explained with the experimental set up. In Khurana and Siddiqi's experiments larvae had two spots on the petri dish where ethanol was offered and the larvae were counted after 2 minutes, while in this thesis ethanol was offered for 5 minutes and only on one side of a petri dish. Another study that was recently published used a similar approach and timeframe like this thesis (Schumann et al.,

2021) and they also showed a Gaussian bell curve shaped concentration dependent decline of attraction. Furthermore, attraction of *CantonS* towards 8% EtOH is also in agreement with prior studies (Schumann et al., 2021). Thus, attraction to ethanol is concentration dependent, on which literature agrees (Khurana and Siddiqi, 2013; Schumann et al., 2021) and appears to be exposure time dependent. This argument is supported by literature, where larvae left 20 % ethanol after some time, but remained on 8 % ethanol containing agarose (Schumann et al., 2021).

Furthermore, *CantonS* approaches up to 10 % ethanol, when it is mixed into the agarose, while w^{1118} leave 10 % ethanol containing agarose. However, w^{1118} larvae remain in proximity of the border between the plain agarose and the ethanol containing agarose half (Figure 15). These results agree with literature (Sumethasorn and Turner, 2016). Here, *Drosophila melanogaster* larvae left ethanol patches after hatching. Lower ethanol concentrations (4 %) were reported to enhance larval fitness and survival, with the most larvae reaching pupation on 4 % ethanol (Schumann et al., 2021) and *CantonS* larvae were reported to crawl on 8 % ethanol containing agarose for up to 120 minutes. Thus, the approach of up to 10 % ethanol in *CantonS* larvae might be due to beneficial effects of low doses of ethanol on larval fitness.

A recent study addressed olfactory memory defects in *Drosophila melanogaster* w^{1118} (Myers et al., 2021). Adult w^{1118} flies exhibit learning deficits after electric shock conditioning, which could be an argument to avoid the w^{1118} genotype for learning and conditioning experiments. However, the learning deficit phenotype was only observed when the electric shock was given only once (and up to three times) in a minute and was no longer observable at higher shocks/minute patterns.

As larvae undergo a complete metamorphosis to become adults as well as behavioural changes, such as odorant attraction in larvae that results in odorant aversion in adult flies, those concerns can be neglected. Furthermore, as w^{1118} larvae are frequently used as control in conditioning experiments (Diegelmann et al., 2013; Ganguly et al., 2020; Selcho et al., 2009; Widmann et al., 2016) where larvae show learning behaviour after conditioning.

Ethanol is attractive, both as an odorant and mixed within a substrate, although in both cases the concentration of ethanol determines whether it is attractive or aversive to the larvae.

Ethanol can be smelled, however, the exact olfactory receptor to which ethanol can bind is still unknown. *Or22a* might be a potential candidate, as was seen by extra-cellular unit measurements with microelectrodes (Hallem and Carlson, 2006; Mansourian and Stensmyr, 2015). Other alcohols are sensed by a wide range of olfactory receptors. For example, 2-phenylethanol is sensed by *Or67b*, 1-hexanol is sensed by *Or35a* and 6-methyl-5-hepten-2-ol is sensed by *Or13a* (Mansourian and Stensmyr, 2015). Therefore, the most likely candidate to bind ethanol cannot be extrapolated from looking at closely related chemical substances. It is likely that ethanol is bound by multiple receptors.

The presence of ethanol changed olfactory attraction to the other tested odorants in a concentration dependent and odorant specific manner in *CantonS* flies (Figure 14). Furthermore, ethanol that was mixed into odorant cups filled with BA elicited aversion in *CantonS* larvae, while w^{1118} larvae showed indifference (Figure 17).

Indeed, it was already reported that certain odorants are known to inhibit basal neuronal firing and that this odour-evoked inhibition drives attraction and aversion behaviour in adult *Drosophila* (De Bruyne et al., 2001; Cao et al., 2017). The *Or85a* expressing neurons fire constantly without a stimulus. However, in the presence of acetophenone firing by the OSN was inhibited (Cao et al., 2017). Depending on the type of OSN, the presence of the odorant acetophenone resulted in either attraction or aversion of certain other odorants. By closing ion channels, acetophenone stops the constitutively active firing of OSNs. Some, like *Or10a* expressing OSNs are thus activated, while others -such as *Or82a* and *Or85a*- are inactivated (Cao et al., 2017).

Neurons expressing *Or22a*, which also binds ethanol, might also be constitutively active. This was proposed by a research group that observed, and published decrease of Ca^{2+} levels because of odorant presentation to these OSNs (Pelz et al., 2006). One of these substances was benzaldehyde, which was one of the odorants that were used as conditioning stimuli.

A similar mechanism could be the reason, why the presence of ethanol decreases the attraction behaviour towards certain odorants in *CantonS* larvae but not in w^{1118}. There is published data on the role of variation within a certain locus of the genome that encodes for olfactory receptors, that result in expression of either *Or22a* or *Or22b* (or *Or22ab*) or both (e.g. in *CantonS)* (Shaw et al., 2019). Adults of these different strains showed different attraction indices to odorants, such as ethyl acetate, pentyl acetate

or 1-octen-3-ol. This could explain, why *CantonS* larvae are affected by the presence of ethanol in their attraction towards odorants, while w^{1118} larval odorant attraction is not affected by the presence of ethanol. There were, up to date, no studies published that addressed a potential link between the w^{1118} mutation and poor or altered olfactory perception in larvae or adults.

Ethanol as a cue for food source availability

Drosophila CantonS larvae show no appetitive memory towards 8% EtOH reinforced odorants, when the test is performed on 8% EtOH or 2 M fructose mixed into agarose (Schumann et al., 2021). *Drosophila* sense fructose over the Gr43a receptor expressing taste neurons (Mishra et al., 2013), therefore the smell of ethanol and the taste of fructose are processed via different neurons, as shown in Figure 16. Taste-information could be more important to the larvae than olfactory-information, which explains why ethanol is no longer approached in the presence of fructose. However, attraction towards odorants, such as AM or 2-Hep, was not suppressed on fructose plates, meaning that taste information is not generally more important than olfactory information.

Adult *Drosophila* show reduced preference for ethanol-containing food in the presence of an isocaloric alternative food source (Pohl et al., 2012). This could explain the lack of attraction towards ethanol in the presence of fructose in larvae.

In the presence of a sugar containing substrate, ethanol is no longer attractive, because the smell of ethanol provides the larvae with information about the availability of nutrients, specifically carbohydrates. This hypothesis is supported by the fact that attraction toward acetic acid also disappears in the presence of 2 M fructose, but the attraction towards ethyl acetate does not. Both odorants are key odorants and present during fermentation processes (Becher et al., 2012; Giang et al., 2017; Piškur et al., 2006), with AA being a key-odorant for presence of *Acetobacter* and EA being a key-odorant for the presence of yeast. Acetic acid, which is formed by oxidization processes of ethanol triggered by bacteria bind to the *Ir75a* receptor (Silbering et al., 2011). Both, ethanol, and acetic acid are attractive odorants to *Drosophila* larvae (Khurana and Siddiqi, 2013; Schumann et al., 2021). Both odorants are also no longer attractive in the presence of sugar (Figure 16), most likely because they are carbohydrate cues.

Larvae and adult flies consume yeast as food source and adult flies prefer yeast as an egg laying ground (Yang, 2018). Indeed, larvae are known to choose a protein-carbohydrate food ratio, that reduces developmental time until pupation (Rodrigues et al., 2015). In adult flies, a recently published study shows significant survival benefits under starvation in the presence of yeast and ethanol volatiles (Luo et al., 2021).
Therefore, larvae and adults show a clear preference for specific food sources that provide significant survival and developmental benefits. It makes sense that potentially redundant information -e.g., smelling sugar fermentation related odorants while sitting in a sugar medium- would be ignored by the larvae to preserve energy by avoiding unnecessary movement. This also explains, why pairing of ethanol with 2 M fructose in conditioning experiments (Figure 16B) does not result in increased attraction towards ethanol, because ethanol loses any internal meaning to the animal in presence of sugar.
It cannot be ruled out completely that larvae are distracted by the presence of 2 M fructose and thus ignore the ethanol smell in conditioning and olfactory preference experiments, but this cannot explain the fact that larvae still show preference towards yeast associated odorants as well as odorants that are not related to alcohol.
Thus, it is highly likely that this loss of preference for ethanol smell on fructose plates is due to alcohol smell being associated with sugar fermentation. Enzymes involved in ethanol and sugar metabolism such as ADH and αGPDH are both -synergistically and individually- upregulated by ethanol and sugar (Geer and Laurie-Ahlberg, 1984; Geer et al., 1983). This further supports the idea that there is a physiological link between both substances.
Larvae can use the carbons from ingested ethanol to synthesise lipids, although when dietary sugars are also present, the organism tends to store those carbons first before utilising ethanol (Geer et al., 1985, 1989). Hence, an internal mechanism must exist, that regulates flux of carbons in lipid synthesis (Geer et al., 1985). Flux appears to be at least partially mediated by the ADH, during the alcohol degradation cascade (Freriksen et al., 1991).

Ethanol is no reinforcer in the tested conditions

Ethanol is a constantly available substrate in nature for *Drosophila* larvae with a lot of beneficial side effects, such as nutrition and protection from predators, and is actively

searched for by adult *Drosophila* as egg-laying substrate (Geer et al., 1985, 1989; McKenzie and Parsons, 1972; Milan et al., 2012; Richmond and Gerking, 1978; Schumann et al., 2021). Although, larvae that hatched on ethanol patches tend to leave ethanol after hatching (Sumethasorn and Turner, 2016).

These published results are consistent with the results presented in this thesis, as most larvae analysed during the side preference assay also left the ethanol containing side within a few minutes but remained close to the border, where both agarose plate halves met, and a low ethanol dose gradient can be expected (Figure 15).

Larvae that started on low dose ethanol sides tended to remain there, which fits nicely with previously published results, where larvae spent up to 120 minutes on low doses of ethanol, with 4 % ethanol even providing a survival benefit to the larvae (Schumann et al., 2021). This can be due to two reasons. One, larvae do indeed prefer to stay on low-dose ethanol. Two, larvae are just indifferent to low doses of ethanol, while they avoid higher doses. The latter theory is supported by the fact, that *Drosophila* can compensate for negative effects of ethanol through inducible *Adh,* which is responsible for alcohol degradation (Geer et al., 1989; McKechnie and Geer, 1984).

Ethanol is, on a cellular level, a toxin (McClure et al., 2011) and has detrimental effects on larval development, often associated with high concentrations (although low concentrations were reported to increase mortality as well) (McClure et al., 2011; McKechnie and Geer, 1984; McKenzie and Parsons, 1972; Ranganathan et al., 1987). Luckily for the larvae, in nature high ethanol concentrations are a rarity (Gibson and Wilks, 1988; Gibson et al., 1981).

Thus, the question was addressed whether naturally occurring concentrations of ethanol could be used as a reinforcer in pavlovian style olfactory conditioning (Aceves-Piña and Quinn, 1979; Pavlov, 1927; Tully and Quinn, 1985) due to its crucial role in the life of a developing larva. Indeed, first results were already published in 2021, where ethanol was successfully utilised as an appetitive reinforcer in olfactory conditioning experiments (Schumann et al., 2021). Larvae showed attraction after three-cycle conditioning, following a well-established protocol (Michels et al., 2017). The results in this thesis are not in agreement with Schumann et al. at most of the tested conditions. This is most likely due to differences in protocol between Michels et al. and this thesis. While Michels et al. and Schumann et al. change the reinforced odorant in the reciprocal group, they also change the point at which the reinforced odorant is presented in the reciprocal group, while this study only altered one condition

per conditioning experiment – the reinforced odorant but not the timepoint of presentation.
Other published studies focused on the effect of ethanol ingestion -chronic and acute- and withdrawal on memory formation in *Drosophila* larvae (Robinson et al., 2012a, 2012b). Here, acute ethanol exposure was shown to result in poor appetitive memory, while chronic ethanol exposure resulted in re-instatement of appetitive memory formation. Chronic ethanol exposure led to ethanol-dependency regarding appetitive memory formation. Furthermore, in adults' ethanol was used as a proper teaching signal (Nunez et al., 2018; Petruccelli et al., 2018).

In adult *Drosophila* repeated exposure to odorants results in habituation and less neuronal activity, while novel odorants are normally met with increased neuronal activity and an alerting state of the animal (Hattori et al., 2017). Furthermore, pre-exposure to odorants was also reported to alter memory formation in adult flies (Jacob et al., 2021). In larvae 5-minute pre-exposure to an odorant -ethyl acetate- resulted in short-term habituation with a half-life time of approximately 20 minutes (Larkin et al., 2010). This short-term habituation depends on GABAnergic inhibition through local interneurons as well as the rutabaga adenylyl cyclase and the choline transporter (ChT) (Hamid et al., 2021; Larkin et al., 2010). Inhibition of the ChT results in hypersensitivity to odorants (Hamid et al., 2021). Coupled with the results presented in Figure 22, where a novel odorant presented during test resulted in attraction, this strongly suggests that the phenotype observed after conditioning is due to habituation and not because of attractive or aversive properties of ethanol.
Larvae show an attraction towards AM after subsequent pre-exposure to AM and BA after one cycle or three cycle in an olfactory conditioning paradigm without reinforcer. BA is reported to be both, an attractant (Bellmann et al., 2010; Khurana and Siddiqi, 2013) and a repellent, when diluted 10^{-2} (Kreher et al., 2008). However, after pre-exposure BA is no longer attractive to the larvae. This shift is probably also a result of habituation, which expresses as a change of odorant valance, due to the presence of two odorants during the testing situation.
When a reinforcer is introduced into the conditioning regime, larval behaviour shifts in a paradigm and concentration dependent manner. In most cases larvae also chose AM after conditioning, independent of the ethanol concentration or genotype of the animals.

One explanation is that ethanol -as an odorant- mixes with BA (or AM) during conditioning or test and is thus re-evaluated as a new, complex odorant. Complex odorants are more attractive than single odorants (Giang et al., 2017; Thoma et al., 2014; Zhu et al., 2003). BA (or AM) might therefore be sensed differently and be less or more attractive. Adult flies prefer ethanol containing odorant over pure odorants (Giang et al., 2017; Schneider et al., 2012). A similar mechanism must be active in *Drosophila* larvae, however the mix of ethanol with an odorant was -in case of AM - meaningless or -in case of BA - aversive.

Indeed, odorant blends are known to also produce inhibitory signals on the level of the antennal lobe (Silbering and Galizia, 2007; Wilson, 2013). Ethanol functions as an odorant. In combination with BA an inhibitory circuit might be triggered.

Kreher et al. reported that, based on distance plot analysis extrapolated from larval behaviour towards certain odorants, benzaldehyde is, however, unlikely to “mask” the presence of another odorant due to its distance in the Euclidean plot (Kreher et al., 2008). In experiments with EA presented as a single odorant point, BA was unable to mask the presence of EA and larvae still approached the EA odorant spot, meaning that both odorants did not blend but were identified as single odorants. Thus, it seems unlikely that BA, even in the presence of ethanol, might be interpreted as a new odorant. This fits with experiments that were performed by Schumann et al. where they show that BA is still smelled and identified by larvae, independent of ethanol presence on the petri dish (Schumann et al., 2021). Ethanol that was mixed into odorant cups filled with BA elicited aversion in *CantonS* larvae, while w^{1118} larvae showed indifference (Figure 17).

Given that BA as an odorant is present in apricot kernels, leaves of *Prunus persica* and bitter almonds (Verma et al., 2017), all of which are not really consumed by *Drosophila* larvae, it is not surprising that it also carries no significant innate meaning to the animal. Mixing attractive odorants with BA was reported to reduce the attraction of the normally approached odorant in adult *Drosophila* and BA is in general characterised as a repellent for adult fruit flies (Thoma et al., 2014).

These results suggest that larvae are indifferent to the presence of ethanol during conditioning. Published results show that larvae do indeed consume ethanol containing medium (Robinson et al., 2012b) and should therefore sense the reinforcer during training. One would expect that a reinforcer that is an integral part of *Drosophila* larval

development in the wild (Geer et al., 1985; McKenzie and Parsons, 1972; Milan et al., 2012; Richmond and Gerking, 1978; Schumann et al., 2021) and is ingested, would affect larval behaviour. However, this thesis clearly shows that ethanol is not generally attractive as a reinforcer. Taken together, in the here tested conditions most phenotypical alterations of behaviour can be attributed to ethanol independent habituation to the odorants.

Alas, the observed effects that are deviating from the reinforcer-less odorant valance shift following pre-exposure are few and most of them are also fleeting. For example, the fact that BA is attractive after conditioning, when BA is paired with ethanol in the first step of every cycle in *CantonS* (Figure 23A). However, this attraction to BA is not observed, when a lower ethanol concentration (5 %) was used in conditioning experiments with *CantonS*, as results produced by Barış Yapıcı (data not shown) show, supporting the labile and fleeting nature of behaviour which deviates from reinforcer-less odorant valance shift.
In literature, learning inhibition induced by acute alcohol exposure (Robinson et al., 2012a) is reversable after some time (Robinson et al., 2012b). Larvae, crawling on ethanol containing substrate, are ingesting the ethanol containing agarose (Robinson et al., 2012a). They might show ethanol induced behavioural alterations, such as appetitive conditioning or, as published by Robinson et al., appetitive learning inhibition. This could be indeed a case, where ethanol is interpreted as an appetitive reinforcer in a conditioning regime dependent manner. However, whether it is intoxication that alters conditioning behaviour, remains uncertain.
For *Drosophila* larvae mean elution times have not yet been measured and it is very likely that no alcohol induced immobilisation might take place in larvae, seeing that at 8% ethanol larvae choose to remain on the ethanol for the whole measured time (120 minutes) -and were reported to still show movement- and even on high concentrations (20 %) larvae only left the ethanol containing side 15 minutes after the beginning of the experiment (Schumann et al., 2021). Larvae left the 20 % ethanol side at some point because this is a dose that is known to significantly increase mortality (David and Bocquet, 1975; Fry, 2001).

Taken together, the results of this thesis support the idea that ethanol under the tested condition is indeed mostly meaningless to the larva. They are attracted to the smell of

ethanol in a concentration dependent manner. The presence of carbohydrates reduces attractiveness of ethanol as an odorant. The attraction to AM appears to be also independent of the ethanol presence. If anything, the presence of ethanol during training suppressed the behavioural shift toward AM after pre-exposure. Therefore, ethanol appears to be not a suitable reinforcer for *Drosophila melanogaster* larvae when performing olfactory associative learning and memory experiments.

The importance of octopamine in memory plasticity and internal state regulated memory formation

The strength of the reinforcer influences appetitive learning and memory

Tβh^{nM18} form aversive memory using 0.15 M sucrose when they were starved for 16 h prior to experiments and their glycogen storage was still highly elevated (Figure 24C). This is surprising, because up until now it was expected that sucrose reward would either produce no memory with 2 M sucrose (Schwaerzel et al., 2003) or reduced appetitive memory with 6 M sucrose reinforcer (Huetteroth et al., 2015).

There are two ways to explain the aversion to a food reward in *Tβh*^{nM18} flies: First, the increased glycogen storage of *Tβh*^{nM18} flies is responsible for 0.15 M sucrose not being attractive. Fitting with this hypothesis is also the observation, that *Tβh*^{nM18} flies show significantly decreased appetite for sucrose in the CAFE assay (Figure 24B).

Second and more likely, the reinforcer is just not strong enough. There is evidence in literature, that the reinforcer intensity has significant effects on olfactory conditioning. The concentration of a reinforcer is known to influence the intensity of the exhibited performance and memory after training for both aversive (Tully and Quinn, 1985) as well as appetitive memory (Colomb et al., 2009; Das et al., 2014; Huetteroth et al., 2015).

Adult *Drosophila* also show dose-dependent behavioural difference after conditioning with ethanol (Nunez et al., 2018). The same conditioning paradigm results in appetitive memory or indifference, depending on how high the reinforcer concentration is.

The results, provided by Nunez et al. also show, how parameters such as concentration of reinforcer or the conditioning paradigm influence memory formation in general. This is an important observation for the experiments of this part of the thesis, as well as the larval conditioning experiments in the previous part of this thesis.

It was also reported that NaCl can act as positive and negative reinforcer in a concentration-dependent manner. In larval learning low doses of salt (0.25 M – 0.3 M) are attractive. This attraction is regulated by the Ir76b gustatory receptor in larvae. Furthermore, low dose salt also functions as an appetitive reinforcer in olfactory conditioning (Russell et al., 2011; Zhang et al., 2013b).

For w^{1118} a dose dependency for sugar reinforced appetitive memory can be observed, seeing that 2 M sucrose produces higher learning scores than 0.15 M sucrose.

Thus, OA signalling shifts the dose response curve for sucrose. To test this hypothesis, w^{1118} flies could be trained with an even lower concentration of sucrose to investigate, whether low dose sucrose would also lead to the formation of aversive memory. It was reported, that $T\beta h^{nM18}$ flies show significantly reduced response to sucrose in comparison to wild type flies, when different concentrations were offered in a modified PER assay (Damrau et al., 2018). $T\beta h^{nM18}$ shows increased glycogen levels (Figure 24C), which could be interpreted as an obesity phenotype. Flies that were raised on high sugar diets also developed an obesity phenotype, which resulted in reduced taste response to sugar (May et al., 2019). May et al. provide evidence, that sweet taste decreases neuronal activity, which results in reduced taste and increased meals. Paired with the observation that OAMB expressing dopaminergic neurons are necessary for sweet taste reinforced STM (Huetteroth et al., 2015), this provides a reason for aversion to 0.15 M sucrose.

Additionally, Damrau et al. also reported that $T\beta h^{nM18}$ flies show slower decreasing haemolymph sugar in comparison to wild type flies, which the authors attributed to higher starvation resistance (Damrau et al., 2018). They also saw that $T\beta h^{nM18}$ survive longer under starvation conditions, which agrees with another publication that analysed starvation resistance in $T\beta h^{nM18}$ (Li et al., 2016).

Taken together, with the results in this thesis, that $T\beta h^{nM18}$ show increased glycogen levels, this reduced response to sucrose (Damrau et al., 2018; May et al., 2019), the necessity of OA signalling onto dopaminergic neurons (Huetteroth et al., 2015) and the apparent shift in dose-response to sucrose as an appetitive reinforcer can be explained with shifts in reinforcer valance due to internal-state dependent changes.

Starvation influences appetitive memory strength

Prolonged starvation is known to alter memory retention and memory strength (measured by increased memory scores) in adult *Drosophila* (Colomb et al., 2009). These results agree with the results obtained in this thesis (Figure 25). Indeed, the results in this thesis also provide further evidence that prolonged starvation results in appetitive LTM formation, which agrees with literature (Colomb et al., 2009).

Robust appetitive LTM is normally produced with a regime relying on repetition with intermediate breaks of 15 minutes, that uses an appetitive reinforcer, such as sucrose (Tully et al., 1994). This memory is regulated by de-novo protein synthesis (Kandel, 2012). Appetitive protein-synthesis dependent, rapidly consolidated memory can also be observed in flies that were trained with 6 M sucrose (Krashes and Waddell, 2008) after only one cycle of appetitive training.

Surprisingly, fasting was reported to not only influence appetitive LTM formation, but aversive LTM formation as well (Hirano et al., 2013). Mild starvation results in protein-synthesis dependent LTM after one cycle of aversive training. It was reported recently that aversive memory, that is formed under conditions of mild starvation (16 h) is maintained by ketone body oxidation, which functions as an alternative energy source for MB neurons (Silva et al., 2022). Nevertheless, aversive STM appears to be unaffected by starvation, while appetitive STM clearly is (Figure 24,25).

Starvation also influences other olfactory behaviour, such as odorant attraction and aversion in larvae (Vogt et al., 2021). Odorants, such as geranyl acetate are normally aversive to fed larvae but become attractive upon starvation (Vogt et al., 2021). Although this thesis did not investigate the effects of changes in olfaction upon starvation, odorant acuity experiments were performed and -at least for $T\beta h^{nM18}$- a decrease in odorant avoidance can be seen upon 40 h starvation (Table 8).

For both, larvae and adult, studies have shown a link between starvation time and memory formation and olfactory behaviour (Brünner et al., 2020; Colomb et al., 2009; Vogt et al., 2021), which fits with the results of this thesis.

Larvae that were starved for 20 h prior to three-cycle fructose reinforced olfactory conditioning showed increased appetitive memory retention, meaning that memory formed after three-cycle conditioning decayed slower (Brünner et al., 2020). This agrees with the observation made in this thesis, seeing that starved flies remembered odorants paired with sucrose for at least 6 h (Figure 24,25).

Starvation was shown to increase cold-shock resistance in larvae (Brünner et al., 2020). A similar observation was made in this thesis for adult flies (Figure 25B). w^{1118}

did indeed form appetitive long-lasting consolidation dependent memory upon 40 h starvation, while *Tβh*^{nM18} might have shifted memory in a similar pattern as was already observed for aversive LTM in starved flies (Plaçais and Preat, 2013), where upon starvation flies shifted aversive memory from energy costly LTM to ARM by abolishing slow oscillation in two pairs of dopaminergic neurons.

This is done in favour of survival and energy preservation, as forced protein-synthesis dependent LTM formation resulted in reduced survival rate in those animals (Plaçais and Preat, 2013). This thesis hypothesises, that energy is invested into costly LTM for appetitive memory in w^{1118} upon 40 h starvation. Whether *w*^{1118} flies show protein-synthesis dependent LTM or protein-synthesis independent ARM is still not completely understood. However, data suggests, that appetitive ARM in adult *Drosophila* might not exist (Colomb et al., 2009).

The publication reported that memory of *CantonS* flies which were starved for 21 h prior to conditioning, was susceptible to CXM treatment in flies that were trained with only one session, as well as 5x massed and 5x spaced training. Normally, CXM treatment only disrupts protein-synthesis dependent memory (Tully et al., 1994). The fact that Colomb et al. show significantly reduced appetitive memory in massed trained flies suggests that *Drosophila* might only form protein-synthesis dependent appetitive LTM and no ARM at all with sugar reward.

The question remains, why *Tβh*^{nM18} flies, that form protein-synthesis dependent memory exclusively, still shift to a rapidly consolidated, anaesthesia-resistant form of memory upon 40 h starvation prior to experiments.

Time between conditioning and cold shock affects the amount of anaesthesia-resistant aversive memory (Quinn and Dudai, 1976; Tully et al., 1994).

Cold shock applied directly after training reduced memory in flies that were trained 10x in massed aversive training to zero (Quinn and Dudai, 1976; Tully et al., 1994). For *Tβh*^{nM18} a defect in aversive ARM was reported, a few years ago (Wu et al., 2013). The authors show that *Tβh*^{nM18} mutants, that undergo a cold shock treatment 2 hours after one cycle of aversive conditioning, show significantly reduced associative memory afterwards. They uncover an important function for octopaminergic signalling through the APL neuron as well as the Octβ2R in aversive ARM formation.

This thesis provides evidence for the existence of an internal-state dependent, rapidly consolidated appetitive memory that is negatively regulated by octopamine and is, by definition, ARM.

The internal state regulates appetitive memory and consumption behaviour

Non-mated virgin w^{1118} and $T\beta h^{nM18}$ flies both show significant appetitive short-term memory using 2 M sucrose as reinforcer. After mating, female flies of both genotypes show no appetitive STM when sucrose is used as reinforcer (Figure 27A). Thus, sugar is no longer a suitable reinforcer to form appetitive associations. This is not surprising, seeing that female flies that carry eggs show increased appetite for amino acids (Corrales-Carvajal et al., 2016; Ribeiro and Dickson, 2010) and show significant yeast preference, when they were either fed with a carbohydrate-only diet before the experiments or when they were mated beforehand. Whether the flies learned anything during conditioning cannot be said, only that they did not exhibit appetitive STM during test.

This is another indicator that the internal state in general significantly affects behaviour such as learning and memory formation. However, $T\beta h^{nM18}$ virgin females still show reduced STM, while male *Drosophila* show no STM at all (Das et al., 2014; Schwaerzel et al., 2003).

Differences in learning between male and female flies are known for the *mbm* genotype, where female flies show poor learning in some learning paradigms (Heisenberg et al., 1985). Therefore, octopamine regulates male and female appetitive STM differently. There is literature, showing different roles of OA in sex specific behaviour. Indeed, sexually dimorphic octopaminergic neurons are known in *Drosophila*, which regulate pre- and post-mating behaviour in female flies (Rezával et al., 2014). Lack of OA was shown to inhibit post-mating behaviour -such as egg laying, ovipositor extension or suppression of courtship and remating-, while OE of OA induced post-mating behaviour in female flies (Rezával et al., 2014). Additionally, OA also regulates courtship behaviour in male flies, which learn to avoid female flies after their advances were rejected (Zhou et al., 2012).

OA was linked as a regulator of starvation induced hyperactivity (Yang et al., 2015). $T\beta h^{nM18}$ flies, as well as flies where OA synthesis was ectopically blocked with Kir2.1 expression, show significantly reduced midline crossing activity to reach a food source in comparison to *CantonS* flies. The authors also saw no general difference for sucrose

consumption between wild type control and *Tβh*^{nM18} under sated and starved conditions, when assayed in a PER assay, which disagrees with other published results (Scheiner et al., 2014). This could be due to different analysed sucrose concentrations, seeing that Yang et al. appear to have worked with lower concentrations.

This thesis saw that lack of octopamine also affects sucrose consumption (Figure 28). In a choice situation the preference for yeast was significantly higher in *Tβh*^{nM18} than in *w*^{1118}. These data, produced by Yang et al. are, on the first look, in agreement with the raw data obtained during this thesis. In a sucrose and yeast choice situation, sated *w*^{1118} and *Tβh*^{nM18} flies show equal amounts of overall consumption (Figure 28A) and show equal amounts of sucrose consumption (data not shown). However, upon starvation this thesis finds difference in consumption behaviour, which disagrees with the published results in Yang et al. This is most likely due to differences in the behavioural approach. The PER and the CAFE assay measure different behaviours that are mechanistically differently regulated.

Furthermore, the shift in sucrose preference does not only agree with the other experiments in this thesis, regarding the internal-state of *Tβh*^{nM18} (increased consumption of yeast, decreased protein levels), literature also agrees with the assumption that the internal-state regulates food choice behaviour.

It was reported that starved flies make trade-offs and choose to consume food that is mixed with bitter substances under starvation conditions, which they normally would not eat when they are sated (Sareen et al., 2021), which is mediated by synaptic gating on the level of the fan shaped body.

Furthermore, another publication already saw a link between changes towards protein consumption preference under starvation (Münch et al., 2022). Flies that starved show significantly more sips of protein food sources, as measured by the flyPAD. They report that protein-deprived flies significantly increase protein consumption, which also fits as an explanation, why *Tβh*^{nM18} flies show a significantly increased consumption of yeast extract in the CAFE assay.

OA signalling is known to regulate hunger and satiety driven shifts in consumption behaviour (Selcho and Pauls, 2019). Diminished neuronal activity of the OA-VL neurons results in reduced bitter sensitivity, while OA-VPM4 activity under starvation is known to enhance sugar sensitivity. Thus, the decreased sucrose preference in

Tβh^{nM18} flies could be the result of three mechanisms: First, the increased glycogen storage. Second: the decreased protein levels. Third: lack of octopamine to enhance sugar sensitivity through OA-VPM4 signalling. Therefore, the primary regulator of learning with yeast reinforcement as well as consumption behaviour is the internal state, altered in the *Tβh*^{nM18} mutants.

Glycogen storage regulates appetitive learning and consumption behaviour

In this thesis the role of the muscles and mushroom bodies as a negative regulator of appetitive memory was uncovered (Figure 29B), seeing that *mef2-Gal4*, the Gal4 driver used to target the muscle, also expresses in the mushroom bodies (Crittenden et al., 2018). However, muscle glycogen appears to primarily function as a starvation monitor, because only upon genetically induced glycogen depletion appetitive memory significantly increased in experimental flies, while an increase of glycogen did not alter memory. However, due to the mushroom body expression it has to be considered, that they are important for the formation of olfactory associative memory (De Belle and Heisenberg, 1994; Heisenberg et al., 1985; Owald and Waddell, 2015). Therefore, learning differences could be attributed to the mushroom bodies.
Furthermore, sucrose consumption improved, when mushroom body expression of the mef2-Gal4 driver line was blocked in *Tβh*^{nM18} (Figure 32). It is very likely, that blocking mef2-Gal4 mushroom body expression in *Tβh*^{nM18} mutant background while knocking down *GlyS* expression would result in improved appetitive 2-min memory. To test this idea, *Tβh*^{nM18}*;mb247-Gal80;mef2-Gal4/UAS-GlyS-RNAi* flies should be tested in appetitive short-term memory conditioning paradigm.

Tβh^{nM18} mutant flies show appetitive STM memory, when glycogen storage was downregulated in muscle and fat body simultaneously (Figure 33).
A study, performed by Wigglesworth in 1949, shows that even after death by starvation glycogen deposits can be found in the flight muscles, while every other organ is properly depleted (Wigglesworth, 1949). Flies tend to exhaust their fat body glycogen first and to a much higher extent (Wigglesworth, 1949). Here, young, 5–7-day old flies, that flew until complete exhaustion, show that flight that was initiated after exhaustion was primarily powered by glycogen that was stored in the fat body (Wigglesworth, 1949). Those exhausted flies showed significantly less glycogen in the fat body than

control flies that were able to fly with intermediate breaks, which implies one of two things.

One, either glycogen storage can be allocated to refill storage that is depleted by flight.

Two, internal mechanisms regulate, that fat body glycogen storage is depleted first.

Both theories suggest that the fat body thus would have a function in common with the mammalian liver (Adeva-Andany et al., 2016), where the liver is a glucose monitor for the whole organism. That the fat body is a major player in the control of energy homeostasis was shown in a publication by Grönke et al. They were able to show that an evolutionary conserved mechanism, regulated by *Lsd2* in a Perilipin-like manner to regulate lipolysis (Grönke et al., 2003) and thus regulating lipid-based energy storage, exists.

In *Drosophila* larvae, the fat body and lipogenesis function as a safeguard for caloric overload, where lethality increases when the fat body levels increase (Musselman et al., 2013; Yamada et al., 2018). Lipogenesis is necessary for larvae to tolerate high sugar diets. Additionally, they show that knockdown of *king-tubby*, which resulted in fat content increase, resulted in reduction of hyperglycaemia.

Yamada et al. were able to show that under starvation, larvae also utilize glycogen in a tissue dependent manner (Yamada et al., 2018).

After 72 h of starvation PAS staining was still colouring the body wall muscles, while the fat body appears to be completely (or at least nearly) blank. All these results above support the hypothesis, that *Drosophila* fat body and the mammalian liver share common tasks. The results in this thesis, regarding regulation of appetitive STM through muscle and fat body could also be interpreted that way (Figure 34).

Glycogen synthase knockdown in both fat body and flight muscle in w^{1118} background resulted in significant upregulation of appetitive STM, while knockdown of glycogen phosphatase, which increased overall glycogen, resulted in significant downregulation of appetitive STM.

That knockdown of *GlyS* in fat body and muscle in $T\beta h^{nM18}$ (Figure 34) probably led to the formation of STM and not rapidly consolidated ARM (as in the case of 40 h starved $T\beta h^{nM18}$ flies) can only be assumed, when looking at the data sets.

To properly test this hypothesis, cold-shock experiments should be performed with these crosses.

Most prior studies primarily focused on regulation of aversive olfactory memory by InR signalling or chico, the InR substrate, in larvae and adult *Drosophila* (Chambers et al., 2015; Eschment et al., 2020; Hirano et al., 2013; Naganos et al., 2012; Tanabe et al., 2017). Blocking InR signalling onto the mushroom body as well as the ellipsoid body resulted in significantly reduced aversive LTM formation (Chambers et al., 2015). In larvae the InR functions as an inhibitor of aversive larval anaesthesia-resistant memory (Eschment et al., 2020). Blocking InR signalling resulted in memory that was not erased by cold shock (Eschment et al., 2020). *Chico* mutants show olfactory learning defects (Naganos et al., 2012), making *chico* expression a necessary requirement for aversive STM.

Insulin-like signalling is also known to be necessary for intermediate-term aversive memory (Tanabe et al., 2017). When *Dilp3* expression is either decreased by aging or RNAi-mediated knockdown, 3 h aversive memory is significantly reduced (Tanabe et al., 2017). All these publications show that the insulin-like peptides and their receptor are necessary for olfactory memory formation. In this thesis, no significant difference in memory formation was observed upon blocking or constitutively activating insulin-like signalling onto octopaminergic neurons in the w^{1118} background (Figure 35A and 35D).

Blocking insulin-like signalling onto *Tdc2-Gal4* positive neurons results in overconsumption in appetite and hunger driven sucrose consumption behaviour (Figure 35B, 35C). When flies whose insulin-signalling was blocked on OA neurons are fed with sucralose prior to 24 h CAFE assays, flies do no longer consume an excessive amount of carbohydrates (Wang et al., 2016) during the test, which appears to contradict the findings in this thesis. Control flies that were fed for 6 days on a sucralose rich diet tend to overconsume on carbohydrates during 24 h CAFE assays (Wang et al., 2016). The authors argue that an imbalance between sweetness and caloric state of the fly simulates a fasting state, that triggers overconsumption (Wang et al., 2016). Octopamine downstream of insulin-signalling is required for the promotion of appetite induced by sucralose feeding (Wang et al., 2016). However, literature in response to Wang et al. was published, which provides an alternative explanation for the observed overconsumption after sucralose pre-feeding (Park et al., 2018). Park et

al. find that sucralose consumption prior to CAFE assays results in malnutrition of animals, which is why they then overconsume. Consumption returns to normal after some time (Park et al., 2018).

Furthermore, it must be taken into consideration that Wang et al. present their data as a difference between control (base line) and sucralose diet group. Thus, an increase in the sucralose diet group is only an increase in relation to the base line, while overall consumption was not shown. CAFE assay analysis in this thesis, however, was done by looking at total consumption per vial and not as a delta between base-line consumption and sucralose diet group consumption. This means that *Tdc2-Gal4* driver flies with InR knockdown could still show overall higher consumption of sucrose, but the difference between base-line consumption and sucralose group is equal.

Therefore, octopaminergic neurons could be responsible for monitoring of the brains energy state, relying on InR function on OA neurons to signal the presence of enough carbohydrate in the system. This appears to be true exclusively for consumption, seeing that InR-signalling block onto OA neurons did not alter appetitive memory formation (Figure 36).

InR signalling inhibits the consumption promoting effects of octopaminergic neurons but does not result in downregulation of carbohydrate ingestion. In wild-type flies, ingestion of carbohydrates and satiety leads to upregulation of unpaired2 (upd2). Unpaired2 signals via GABAergic neurons, which are inhibitory neurons, to the IPCs (Rajan and Perrimon, 2012). The unpaired2 signalling relieves the inhibitory tone of the GABAergic neurons, resulting in release of Dilps from the IPCs and import of carbohydrates into cells.

Due to the fact, that $T\beta h^{nM18}$ mutants show significantly increased levels of glycogen, the organism is in a satiated state which leads to unpaired2 mediated Dilp release and which could explain the significantly lower levels of haemolymph sugars that were reported in another study (Li et al., 2016).

This also suggests that insulin-like signalling through octopaminergic neurons might function as a back up to regulate appetitive STM in $T\beta h^{nM18}$ mutants, as this study uncovered that expression of UAS-InR^{dn} was sufficient to induce appetitive short-term memory in $T\beta h^{nM18}$ mutants (Figure 37A).

In $T\beta h^{nM18}$ mutant background, where glycogen is not as strongly depleted after 16 h starvation, the organism is not yet in a hunger state. However, InR block on OA neurons by InR^{dn} overexpression, triggers hunger and thus results in appetitive STM

formation in an internal-state dependent manner. *Tβh*^{nM18} mutants that are starved for 16 h form appetitive protein-synthesis dependent memory exclusively. These findings are consistent with a study for aversive learning (Naganos et al., 2012; Tanabe et al., 2017) where *chico* mutants were shown to regulate memory formation but not memory retention or retrieval and dilp3 was shown to be important for aversive memory maintenance but not for memory acquisition.

Furthermore, insulin-like signalling is necessary for the formation of protein-synthesis dependent aversive memory (Chambers et al., 2015). However, appetitive long-term memory appears to be independent of insulin-like signalling. The data are consistent with aversive and appetitive memory, because both memory forms are molecularly distinct (Cervantes-Sandoval and Davis, 2012; Perisse et al., 2016; Schwaerzel et al., 2003).

Octopamine functions as a switch between memory phases

"Emerging" appetitive memory was reported in *Tβh*^{nM18} (Huetteroth et al., 2015). The emergence of this memory is related to consolidation time dependent delay. If the internal state alone was the driving force of memory plasticity and behavioural adaptation, octopamine-less mutant flies would not show LTM formation under glycogen rich storage conditions. This thesis provides evidence that octopamine signalling functions like a switch to guide learned information between different forms of memory (Figure 38).

Prior reports show that OA feeding to *Tβh*^{nM18} flies rescues appetitive short-term memory defects (Schwaerzel et al., 2003). Alas, the report primarily focused on appetitive short-term memory and not long-term memory.

A similar role for OA was found in another behavioural paradigm: OA also functions as a switch regarding food choice behaviour (Claßen and Scholz, 2018) as well as post-mating behaviour in female flies (Rezával et al., 2014). Octopaminergic signalling in specific neuronal subsets lead to complete aversion or attraction of the same food source (Claßen and Scholz, 2018).

The authors show that this behavioural switch appears to be suppressed in *Tβh*^{nM18} mutant flies. Normally flies show attraction to ethanol containing food sources but do not choose ethanol containing food when they lack octopamine (*Tβh*^{nM18} mutant flies) (Claßen and Scholz, 2018; Schneider et al., 2012). This lack of attraction can be

modified when flies are pre-exposed to ethanol. Feeding OA to *Tβh*^{nM18} also restored attraction to ethanol (Claßen and Scholz, 2018). Interestingly, feeding an OA-receptor agonist to control flies resulted in reduced ethanol attraction in control flies. The authors suggested that prolonged octopaminergic signalling might result in negative attraction at the end.

When *w*^{1118} flies were fed OA prior to the conditioning experiments they show significantly reduced STM 2 minutes after training (Figure 38B), which could be explained with the observations made by Claßen and Scholz. However, seeing that feeding OA for even longer did not alter appetitive STM, this explanation might not be suitable for learning and memory formation.

Octopamine might function in a similar manner to *dunce* which provides a check point to inhibit unnecessary formation of costly LTM, by inhibiting dunce in SPN, newly characterised serotonergic neurons (Scheunemann et al., 2018). RNAi-mediated knockdown of *dunce* resulted in LTM formation after short-term memory conditioning (Scheunemann et al., 2018). If octopamine is lacking, memory is directed towards LTM and with increase of octopaminergic signalling at certain time points during memory consolidation, this memory might be inhibited from being consolidated.

Furthermore, it was reported that signalling onto *Oct1βr* in either PNs or MB *αβ* lobe neurons regulates approach or avoidance of a CS+, respectively (Sabandal et al., 2020), which fits very well with the results of this thesis and the idea that octopaminergic signalling functions as a behavioural monitor depending on signalling frequency, time point or which neuronal subsets release the neurotransmitter at a certain point. This supports the thesis that OA is not only a feeding behaviour regulator but regulates associative learning.

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Data Availability Statement

All raw data that was produced by me during my time as a PhD student was given to Prof. Dr. Henrike Scholz for storage and save keeping. Raw data was saved on a DVD and an additional hard drive copy. When necessary, data can be accessed through Prof. Dr. Henrike Scholz.

List of Abbreviations

1-oct = 1-octanol
2-hep = 2-heptanone
3-oct = 3-octanol
5-HT = 5-hydroxytryptamine
AA = Acetic acid
Adh = Alcohol dehydrogenase
Akh = Adipokinetic hormone
AkhR = Adipokinetic hormone receptor
AL = Antennal lobe
Aldh = Aldehyde dehydrogenase
AM = Amyl acetate
AMP = Adenosine mono phosphate
ARM = Anaesthesia-resistant memory
AS = Amyloglycosidase
ASM = Anaesthesia-sensitive memory
BA = Benzaldehyde
BSA = Bovine serum albumine
cAMP = Cyclic-adenosine mono phosphate
CREB = cAMP responde element-binding protein
CS = Conditioned stimulus
Cxm = Cycloheximide
DAN = Dopaminergic neuron
DILP = Drosophila insuline-like peptide
DSK = Drosulfakinin
DTK = Drosophila tachykinin
EA = Ethyl acetate

EtOH =Eethanol

GABA = γ-Aminobutryic acid

GlyP = Glycogen phosphatase

GlyS = Glycogen synthase

GR = Gustatory receptor

h = Hour

InR = Insulin-like receptor

InRca = Insulin-like receptor constitutively active

InRdn = Insulin-like receptor dominant negative

IPC = Insulin-producing cells

ITM = Intermediate-term memory

KC = Kenyon cell

LI = Learning index

Lsd2 = Lipid storage droplet-2

LTM = Long-term memory

MAPK = Mitogen-activated protein kinase

M = Molar

MB = Mushroom body

MBIN = Mushroom body input neurons

MBON = Mushroom body output neurons

MCH = 4-methyl cyclohexanol

min = Minutes

MTM = Middle-term memory

NaCl = Sodium chloride

OA = Octopamine

OAMB = Octopamine receptor in mushroom bodies (one certain receptor type)

OAN = Octopaminergic neuron

OA-VL = Octopaminergic ventral lateral

OE = Overexpression

OR = Olfactory receptor

ORN = Olfactory receptor neuron

PAS = Periodic acid Schiff

PBS = Phosphate buffer saline

PER = Proboscis extension response

PI = Performance index

PKA = Protein kinase-A

RNAi = Ribonucleic acid interference

STM = Short-term memory

Tdc2 = Tyrosine decarboxylase 2

TfAP2 = Transcription factor AP-2

Tβh = Tyramine-β-hydroxylase

Upd2 = Unpaired 2

US = Unconditioned stimulus

VPM4 = Ventral paired median 4

VUM = Ventral unpaired median 1

Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

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Publications

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Knabbe, J., Protzmann, J., Schneider, N., **Berger, M.**, Dannehl, D., Wei, S., Strahle, C., Tegtmeier, M., Jaiswal, A., Zheng, W., Krüger, M., Spanagel, R., Bilbao, A., Engelhardt, M., Scholz, H., & Cambridge, S. B. (2022). Single-dose ethanol intoxication causes acute and lasting neuronal changes in the brain. Proceedings of the National Academy of Sciences, 119(25), e2122477119. (All data published in this manuscript is not part of this thesis)

Berger, M., Yapıcı, B., & Scholz, H. (2023). The function of ethanol in olfactory associative behaviors in Drosophila melanogaster larvae. Plos one, 18(3), e0276714. (shared co-first authorship)

Berger, M., Auweiler, K., Tegtmeier, M., Dorn, K., El Khadrawe, T., Scholz, H., Octopamine integrates the status of internal energy supply into the formation of food-related memories. eLife (Submitted – currently accessible as a reviewed preprint, October, 2023).

Acknowledgments

Here we are.
At the end of the line. I made it. But I did not arrive here on my own. Many people (and even more flies, when we count the individual) helped me on my journey, and I am about to thank everyone personally. So, strap in.

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