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Abstract

To investigate the natural variation of behavioral traits such as learning and perceptual preferences researchers require methods simple enough to test large groups of individuals and sensitive enough to overcome the variability of behavioral traits. To this aim, during my master project I have developed and tested a new method to investigate olfactory learning and olfactory preferences in fruit flies (*Drosophila melanogaster*).

Using this new method I showed a significant olfactory learning performance after a simple conditioning procedure in a large population of flies derived from a natural population and in several inbred lines. Moreover, I investigated the spontaneous preferences for orange odor compared to apple odor in a wild population and inbred lines, showing that the method is sensitive enough to detect spontaneous preferences for orange odor compared to apple odor. Hence this method proved to be simple and sensitive enough to condition large groups of *D. melanogaster* and single inbred lines on olfactory learning and determine their spontaneous preferences for different odors.

In this thesis I discuss how this behavioral method can be used to investigate also different sensory modalities and domains such as vision and spatial orientation. An interesting possibility to apply this method is the phenotyping of large groups of flies in the context of experimental evolution studies, including the evolve and resequence approach (E&R), and genome-wide association studies (GWAS).

List of abbreviations

US	unconditioned stimulus
CS	conditioned stimulus
UR	unconditioned response
bp	base pairs
STM	short-term memory
MTM	middle-term memory
LTM	long-term memory
l-LTM	long-lasting long-term memory
ATM	anesthesia-resistant memory
GWAS	genome-wide association study
SA	South Africa
EMS	ethyl methanesulfonate
MB	mushroom bodies
LH	lateral horn
ORN	olfactory receptor neurons
CREB	cAMP response element binding protein
CXM	protein synthesis inhibitor cycloheximide
PKC	protein kinase C
E&R	Evolve&Resequence

1 Introduction

1.1 Associative learning

"Learning and memory allow an individual to develop an adaptive behavioral response to a novel situation, even one never encountered in the evolutionary past of the species" (Mery et al., 2007). For this reason learning can increase the fitness of an individual and is an important topic of research in evolutionary biology and genetics (Mery and Kawecki, 2002; Mery and Kawecki, 2003)

Learning has been observed in species as diverse as nematodes (Wen et al., 1997), insects (Dukas and Bernays, 2000), rodents (Tryon, 1940) and human beings (reviewed in Hall, 1936). The nematode *Caenorhabditis elegans* is able to learn the association between an ion A with appetitive food and an ion B with aversive garlic extract (Wen et al., 1997). *Drosophila melanogaster* can learn to associate an odor with aversive or appetitive food (Mery and Kawecki, 2002). Rats are able to perform spatial learning in a maze (Tryon, 1940). In this experiment rats learned to navigate through a maze to obtain a food reward. Furthermore rats exhibit rapid conditioned food aversion (Garcia et al., 1955). In this study rats drank sweetened water, and after this event researchers exposed them to radiation, that caused sickness. Rats associated the flavor of the sweetened water with the sickness and in subsequent tests avoided sweetened water.

The association between two stimuli, or a behavior and a stimulus, is called **associative learning**. It is possible to measure whether an individual learns an association using behavioral responses. A simple form of associative learning is **classical conditioning** or Pavlovian conditioning, after the physiologist who first studied this phenomenon. Pavlov used dogs as a model organism. He discovered that an unconditioned stimulus (US) such as food, that elicits an unconditioned response of salivation, can be linked to a conditioned stimulus (CS) such as the sound of a bell. While the food elicits a salivation response in the dog, initially the sound of the bell does not produce any salivation in the dog. After several associations, in which a bell immediately before the food is presented to the dog, the salivation of the dog in response to the bell is measured, without presenting any food (Pavlov, 1927). After conditioning, the dog salivates at the presentation of the sound

of the bell, even in the absence of food. Hence the dogs are able to associate the sound of a bell (CS), with food (US).

A similar approach can be used with different stimuli and species to investigate the learning capabilities of different species in different domains. For instance it is possible to use odors and flavors as conditioned and unconditioned stimuli in fruit flies (Mery and Kawecki, 2002; Zrelec et al., 2013). If an odor is consistently associated with appetitive food one expects that flies learn to associate this odor with the appetitive stimulus, whereas if a specific odor is consistently associated with an aversive food, one expects that flies learn to avoid the odor associated with the aversive stimulus.

Another form of associative learning is **operant conditioning,** first investigated by the psychologist Skinner (e.g. Skinner, 1948). Operant conditioning is based on reinforcement and punishment of behaviors in response to specific stimuli. For instance animals can learn that pressing different levers is associated with different outcomes. In a Skinner box, rats are able to learn the association between pushing lever A with retrieving food (positive reinforcement) and pressing lever B with retrieving a loud noise or shock (positive punishment) (Skinner, 1948).

Associative learning can be studied using different paradigms. The T-maze assay is well established to study the olfactory learning performance (Helfand and Carlson, 1989; Ai et al., 2010). In a classical conditioning assay flies are alternatively exposed to two different odors and learn to associate an odor with an aversive stimulus (Tully and Quinn, 1985). In presence of odor A, flies receive electrical shocks, but odor B is not associated with electrical shocks. After several trials in which flies are exposed to odor A together with electrical shocks, and odor B without electrical shocks, flies are transferred in a decision chamber (a T-maze). Conditioned flies avoid the odor previously associated with electrical shocks showing that they can remember the odor previously associated with punishment.

Another assay uses the proboscis-extension reflex (PER). PER is an unconditioned response after sucrose presentation on the tarsi (Nelson, 1971). Female *D. melanogaster* can learn to associate a sugar reward with a tone (Menda et al., 2011). Shortly after presence of the tone, the researchers presented water supplemented with sugar at the foreleg tarsi, causing a PER. After several trials in which flies were

exposed to the tone and the sugar water, flies associated the tone with the reward, which caused a PER when they were exposed to the tone, even without presentation of sugar water.

A more naturalistic learning assay based on associative learning makes use of free ranging flies are conditioned to associate odors with appetitive or aversive taste (Mery and Kawecki, 2002). This method is called oviposition paradigm. During the first period (3 hours), free ranging flies were exposed to two different foods offered in Petri dishes. In this exposure period, one Petri dish contained pineapple medium and the other orange medium supplemented with the bitter substance quinine (and vice versa in other groups of flies). Flies could learn to associate either pineapple or orange odor with appetitive taste in a single trial. During the test period flies were expected to avoid ovipositing on media associated with aversive taste during the first period. After some generations of selection, flies showed a significant higher proportion of eggs laid in the right medium during the second and third period. More recently a similar method was used in a semi-natural environment (a greenhouse), and flies showed to be able to learn in the absence of selection (Zrelec et al., 2013).

Flies can also learn through operant conditioning, for instance to keep the leg flexed to avoid an electric shock (Booker and Quinn, 1981). In the Horridge's paradigm (Horridge, 1962) individual flies were placed above an electrolyte solution and were only able to move one leg. When a wire tightened on the fly's leg extended into the solution, an electric shock was released. The fly learned to keep the leg lifted to prevent an electrical shock.

In another operant conditioning method, single flies were conditioned to avoid a part of a chamber (Wustmann et al., 1996). When the fly entered a specific part, it was heated up. After some trials, in which the specific part got associated with heat, flies avoided to enter the heat-associated part even when it was not heated due to memory formation.

In my thesis I focus on olfactory conditioning in *D. melanogaster*, trying to improve methods used to phenotype associative learning in this species. In fact *D. melanogaster* is currently used as a model organism for several reasons. First, compared to other eukaryote organisms the maintenance of large numbers of fruit flies is cheap and easy to handle. The morphology can be studied easily using

anesthesia (e.g. CO_2 gas). Flies have a short generation time that enables scientists to perform artificial selection and experimental evolution studies. It requires only 10 days to obtain a new generation at 25°C, and 21 days at 22°C.

Second, the complete genome has already been sequenced and annotated (reviewed in Adams et al., 2000). The genome is relatively small and consists of about 123,000,000 base pairs (bp) encoding for approximately 15,000 genes on three autosomes and one sex chromosome. Due to the large amount of genetic information and tools available, genetic studies in *D. melanogaster* are easier than for non-model species. Hence *D. melanogaster* is a good model to investigate the genetic and genomic base of learning.

Moreover, *D. melanogaster* is already one of the most important models for the study of memory and learning (reviewed in Dubnau and Tully, 1998, Quinn et al., 1974). In the literature it has already been shown that fruit flies are a good model to investigate olfactory conditioning. In fact fruit flies promptly learn the association between an odor and an appetitive or aversive stimulus (reviewed in Busto et al., 2010; Davis, 2005).

1.2. Relevance of *D. melanogaster* in the study of learning and memory

There are many advantages of using flies as a model for learning. First the learning performance in different sensory modalities can be measured easily, for instance olfactory classical conditioning is fast in fruit flies (reviewed in Busto et al., 2010). Second, it is simple to maintain large groups of flies under the same environmental conditions, thus reducing the uncontrolled environmental variability that usually affects behavioral studies.

In some cases flies are a good model due to their similarity to vertebrates. In the last decades scientists have focused particularly on the investigation of olfactory learning, due to the existence of many similarities in the function and anatomy of the olfactory nervous system between insects and vertebrates (reviewed in Davis 2004).

Fruit flies can also be used to test evolutionary hypotheses. One hypothesis that attempts to explain why we observe variability in learning capabilities (Mery and Kawecki, 2002) is that there is a trade-off between fitness advantages and costs

(reviewed in Mery, 2013). Scientists that measure advantages and costs for learning support this hypothesis. After seeing parasitic wasps, *D. melanogaster* changed its ovipositing behavior and laid eggs in food containing ethanol, which led to fitness advantages (Kacsoh et al., 2013). Furthermore social learning in *D. melanogaster* larvae is supported because larvae were able to learn the association between high-quality food with a spatial accumulation of other larvae (Durisko and Dukas, 2013).

Other experiments revealed fitness costs related to learning due to segregating variation during evolution (Mery and Kawecki, 2003; Dukas, 1999; Nepoux et al., 2010). Selection of *D. melanogaster* for improved learning showed a fitness disadvantage in larval competitive ability compared to control flies. This indicates the presence of constitutive fitness costs for improved learning ability (Mery and Kawecki, 2003). Moreover the lifetime of *D. melanogaster* was shorter in flies selected for improved learning compared to unselected control flies (Burger et al., 2008). Later in life, flies selected for enhanced learning show a minor decrease of fecundity (Burger et al., 2008). Another study suggests that flies induced to form LTM have a decreased resistance to extreme stress like desiccation and starvation compared to control flies (Mery and Kawecki, 2005). Furthermore *D. melanogaster* larvae show a better developmental rate, survival rate and adult dry body mass when they were raised in isolation on law quality food, compared to social learning larvae that are raised in groups on high-quality medium, due to increased competition fitness costs (Durisko and Dukas, 2013). Summarizing, the learning capabilities in fruit flies are suggested to be constrained based on the trade-off between fitness advantages and costs of learning.

D. melanogaster has been used as a model for neurobiological studies of learning and memory as well. Recently scientists have used *D. melanogaster* to shed light on the formation of memory and the neuroanatomic substrates of learning. Memory formation requires a neuronal network that is able to receive sensory input to mediate the resulting behavior. Starvation prior to a single-cycle conditioning favors formation of appetitive long-term memory (LTM), but starvation disables formation of aversive LTM. This is due to the fact that in nature starving flies are rewarded when they find nutritious food, which suggests that the new energy leads to LTM formation (Plaçais and Preat, 2013). Scientists have developed an approach, in which they have

screened for mutants with an abnormal brain structure, after inducing mutagenesis with chemicals. With this approach, it has been discovered that the mushroom bodies (MBs) are important for olfactory learning (Heisenberg et al., 1985). MBs are located in the brain and olfactory information is delivered from olfactory neurons (ORN), expressing at the antennae, through different nerves to the MBs (reviewed in Davis, 2005). The importance of MBs for learning and memory is confirmed by the expression pattern study of *dunce* mutants. *Dunce* is a gene important for olfactory learning and memory (Dudai et al., 1976), due to protein expression in the MBs (Nighorn et al., 1991). Furthermore studies on fruit flies revealed that MBs neurons are able to distinguish different odors and their concentrations (Wang et al., 2004). MBs are also used for courtship song learning in male *D. melanogaster*, by modulating the mating behavior through dopamine neurons (Keleman et al., 2012).

Studies on *D. melanogaster* are important also for biomedical research and have implications for human related diseases. Associative learning and memory are widespread across species and thus new findings are potentially relevant for other species, including human beings. In particular, *D. melanogaster* is already used as model for the study of neurodegenerative diseases like Alzheimer disease and Parkinson's disease (Lenz et al., 2013).

1.2.1 Memory types and memory mutants in *D. melanogaster*

Different types of memory have already been studied in flies: short-term memory (STM), middle-term memory (MTM) (also called intermediate-term memory), anesthesia-resistant memory (ARM) and long-lasting long-term memory (l-LTM). The existence of these memory stages and their independence has been shown in two ways: (a) by using the mutagenic approach and finding mutants that are selectively impaired in one memory stage, in contrast to wild type flies (reviewed in McGuire et al., 2005); (b) by identifying memory stages that are differently affected by the inhibition of protein synthesis (Yin et al., 1994).

The duration and resistance of memory depends on the used conditioning procedure (reviewed in Dubnau et al., 2003). Short-term memory (STM) is observed after conditioning and lasts 30 minutes (Tully and Quinn, 1985). The existence of this transient memory stage was confirmed by the impaired performance of *dunce*

mutants in comparison to wild type flies. Wild type flies and *dunce* mutant flies were conditioned to associate an odor with electric shocks. In wild type flies STM lasts 30 minutes after training whereas *dunce* mutants have no STM. STM is protein synthesis independent, because it can't be blocked by protein synthesis inhibitors (Tanaka et al., 2007).

Middle term memory (MTM) is observed between 30 minutes and 3 hours after conditioning (Guan et al., 2011). The existence of this memory stage has been revealed using reversal learning experiments (Tully et al., 1996). In this assay flies were first conditioned to avoid odor A and after some retention time, flies are conditioned to avoid odor B. *Amnesiac* mutants showed a lack of MTM formation compared to control wild type flies.

Two forms of long term memory (LTM) have been described: (Huang et al., 2012): anesthesia-resistant memory (ARM) and long-lasting LTM (l-LTM).

Anesthesia resistant memory (ARM) is observed directly after massed conditioning, in which flies are conditioned during ten conditioning intervals without included rest intervals. This kind of memory can last for days depending on conditioning intensity (Quinn and Dudai, 1976). In an olfactory learning assay, a *D. melanogaster* population is conditioned to associate an odor with electric shocks using massed conditioning. After training, flies were anesthetized with cold shock for different time periods. In this way it was possible to test the memory performance after different delays. Memory became more resistant to anesthesia 10 to 30 minutes after training. *Radish* mutants have a selective impairment in ARM formation three hours after training, compared to wild type flies (Folkers et al., 1993).

Long-lasting LTM (l-LTM) is induced by multiple spaced conditionings, which consist of multiple conditioning trials interrupted by rest intervals. L-LTM can last longer than ARM up to one week (Tully et al., 1994). Differently from ARM, l-LTM is protein synthesis dependent because it is disrupted by the protein synthesis inhibitor cycloheximide (CXM) (Tully et al., 1994). This suggests that ARM and l-LTM belong to two independent memory circuits (Tully et al., 1994).

Concluding, the mutagenic approach is a very useful tool to investigate the different memory stages, studying the learning performance of mutants in contrast to wild type flies.

1.2.2 Genetic basis of learning in *D. melanogaster*

Mutagenesis experiments show that in fruit flies memory and learning have a genetic component (reviewed in McGuire et al., 2005; Dubnau et al., 2003). The main methods used are the study of mutants (Dudai et al., 1976; Folkers et al., 1993) and selection for improved learning (Mery and Kawecki, 2002).

Mutagenesis can be induced by chemicals or by inserting P-elements into the genome. As for mutagenesis induced by chemicals, the mutagen ethyl methanosulfonate (EMS) is used to produce single gene defects. Screening the mutants has allowed researchers to discover genes important for learning (reviewed in Aceves-Pina et al., 1983). Using the mutagenic approaches it was possible to identify several genes important for learning and memory (e.g. *dunce, rutabaga, radish, cabbage, turnip, amnesiac, latheo, linotte* and *foraging*). This approach has enabled to discover *dunce* as the first memory mutant (Dudai et al., 1976). **Dunce** is necessary for STM formation (Dubnau and Tully, 1998). Investigating the *dunce* mutant has revealed the importance of MBs for learning and memory (Nighorn et al., 1991). The **rutabaga** mutant plays a role in STM formation (Dubnau and Tully, 1998). Similar to *dunce*, *rutabaga* is particularly expressed in the MBs, providing more evidence of the MBs' importance for memory formation (Han et al., 1992). Another gene that has been discovered with chemical mutagenesis is **cabbage** (Aceves-Piña and Quinn, 1979). *Cabbage* mutants are not able to associate odors with electric shock, thus they can't perform olfactory learning.

Another method induces mutagenesis by introducing P-elements into the genome. The advantage of this method is that one can define the exact location of the P-element easily using a P-element carrying plasmid and inverse PCR (reviewed in Tully et al., 1990). The P-element destroys the gene function; nevertheless chemical mutagenesis is more efficient. Using the P-element insertion mutagenesis has identified **latheo** as a learning and memory mutant (Boynton and Tully, 1992). *Latheo* is located at the synapse of motor neurons and is used for synaptic transmission (Rohrbough et al., 1999). Another gene that has been identified with the P-element insertion mutagenesis is **linotte** (Dura et al., 1993). *Linotte* mutants show abnormal brain structure (Simon et al., 1998) and defects in MBs (Moreau-Faouvarque et al., 1998). These and more mutants are further described in Table 1.1.

Table 1.1. Important genes used for learning and memory in *D. melanogaster*

dunce	Necessary for olfactory learning and STM formation (Dudai et al., 1976) and *dunce* mutations are suggested to produce a defect in cAMP phosphodiesterase activity (Byers et al., 1981).
rutabaga	Important for the activity of a Ca^{2+}/calmodulin-sensitive adenylyl cyclase (Livingstone et al., 1984) and *rutabaga* mutants do not have associative STM formation (Zars et al., 2000).
radish	*Radish* mutants do not have ARM formation (Folkers et al., 1993).
cabbage	*Cabbage* mutants are impaired in olfactory learning (Aceves-Piña and Quinn, 1979).
turnip	Needed for olfactory learning (Aceves-Piña and Quinn, 1979) and *turnip* is supposed to be necessary for activation of protein kinase C (PKC), which is important for olfactory learning (Choi et al., 1991).
amnesiac	Important for STM formation (Quinn et al., 1979) and a*mnesiac* shows a defect in MTM formation (Tully et al., 1996) too.
latheo	*Latheo* mutants are impaired in synaptic transmission (Rohrbough et al. 1999).
linotte	*Linotte* mutants show a developmental defect in brain structure (Simon et al., 1998).
foraging	Important for olfactory learning in larvae, it affects the cGMP-dependent protein kinase (PKG) (Kaun et al., 2007; Mery et al., 2007) and *foraging* is required for operant visual learning (Wang et al., 2008). Two allelic variants of the *foraging* gene, *for^R* (rovers) and *for^S* (sitters) are identified in natural populations of *Drosophila melanogaster* (Sokolowski ,1980).

Most of the learning and memory mutants investigated so far have been produced by human-induced mutagenesis. A notable exception is the ***foraging*** gene, which has been discovered analyzing the behavior of larvae in a natural population (Sokolowski, 1980). Genetic polymorphisms in the *foraging* gene lead to different performances of memory formation (Mery et al., 2007). The allelic rover variant *for^R* showed a better STM formation, but a worse LTM formation compared to the homozygous sitter variant *for^S* in adults. These natural alleles affecting behavior indicated that flies moving more around (rovers) profit from fast learning, whereas sitting flies (sitters) profit from longer learning. Similar to adults, larvae with the rover allele moved significantly more while eating, compared to larvae with the sitting allele (Pereira and Sokolowski, 1993).

More recently developed methods can help us investigating the natural variation associated with learning in natural populations. With a genome-wide-association approach (GWAS) (reviewed in Wang et al., 2010) it would be possible to investigate if mutants for learning and memory occur in wild populations.

Due to the fitness disadvantages of the artificially induced mutants, it is very unlikely to observe them in natural populations. Using a GWAS would further enable us to study natural variation in learning performance, possibly to discover new causative variants that affect learning.

Due to the **trade-off** between advantages and disadvantages of learning we expect to find variability associated to learning (reviewed in Mery, 2013). Experimental evidence confirms the presence of variability for learning in fruit flies (reviewed in Kawecki, 2010; Lofdahl et al., 1992). Experimental evolution studies on fruit flies have confirmed that natural populations have sufficient variation in the genetic component of learning, to enable selection for enhanced learning (Mery et al., 2007; Mery and Kawecki, 2002). Mery and Kawecki (2002) showed that flies selected for enhanced olfactory learning, perform significantly better after some generations, using the oviposition paradigm.

Information on the heritability and genetic architecture of learning comes from the response to selection of populations exposed to experimental evolution for enhanced learning through laboratory natural selection (Mery and Kawecki, 2002) and artificial selection (Brandes, 1991). Learning abilities have a heritable component as the

strong genetic variability of related and unrelated honeybees supports (Brandes, 1991). Using PER conditioning researchers have selected honeybees for high and low PER scores. The change in performance of the F1 generation shows that there is segregating variation in the parental line (Brandes, 1991).

Experimental evolution studies in *D. melanogaster* showed that there is enough variation in natural populations of flies, to select for enhanced olfactory learning (Mery and Kawecki, 2002; Mery et al., 2007). This study was based on the **oviposition paradigm** (Mery and Kawecki, 2002). In the next paragraph I describe the details of this paradigm.

An aversive substance (quinine 4g/L) was added to either orange or pineapple medium. During three periods of 3 h, 150 flies from each of eight experimental and eight control populations, were exposed simultaneously to an orange and a pineapple media located in petri dishes. Generation 1, 3, 5 and subsequent odd generations were exposed to orange as appetitive stimulus and pineapple as aversive stimulus. Generations 2, 4, 6 and subsequent even generations were exposed to pineapple as appetitive stimulus and orange as aversive stimulus.

In the first period (exposure) even-numbered experimental generations had a chance to associate orange with aversive taste and pineapple with appetitive taste. In the subsequent test periods no aversive cue was present in the media. Good learners were expected to avoid ovipositing on media associated with an aversive substance more often than bad learners, even when the aversive substance had been removed from the medium. Experimenters propagated only eggs that were laid on media never associated with quinine. This procedure was repeated for 57 generations.

Between generation 15 and generation 27, the researchers observed that populations selected for learning laid a significantly higher proportion of eggs, in the medium never associated with quinine compared to control populations within 2 hours after the removal of quinine. The same trend was observed between 2 and 4 hours after the removal of quinine, but the effect was less pronounced, suggesting that the memory of flies decayed after time or that flies learned that quinine was not anymore present in media. In this experiment most of the evolutionary change occurred within the first 20 generations.

This experiment showed that learning capabilities can evolve in a natural fly population, indicating that standing genetic variation of learning is present in the fly population. Nevertheless the paradigm used has also some disadvantages. Using this method experimenters can select only for olfactory learning in females, but not in males, because eggs were selected only based on female performance, thus reducing the population size on which selection is imposed. Furthermore this procedure is very time consuming because the eggs have to be washed every generation before the propagation on the standard medium. Eggs were developed on standard medium to avoid a bias of the preference regarding orange or pineapple media. Further serious disadvantages are that researchers select not only for improved learning performance, but also for fast egg laying. In fact only eggs laid during the short time of the testing phases (6 hours) were used for the next generation. Moreover, this procedure introduces also selection on resistance to egg washing. These drawbacks impose severe limits on the possibility to use the oviposition paradigm to identify causative variants important for learning. In fact, although the evolutionary response indirectly shows the presence of genetic variation for learning the described study did not investigate the genomic change induces by selection for enhanced learning.

The need to propagate and phenotype groups of flies large enough to produce reliable and fine-scale genomic data have been recently described in Kofler and Schlötterer (2013). For these reasons the oviposition paradigm is not appropriate for genomic studies based on the learning performance.

Hence it would be necessary to develop an improved method that can enable researchers to run experimental evolution studies that can be conducted on larger samples, with less effort and selecting only on learning performance. Such a method would open the possibility of genomic investigation of the genetic basis of learning. In my thesis project I worked on establishing a method suitable for large scale genomic studies.

1.2.3 Behavioral studies of associative learning in fruit flies

At the behavioral level, in fruit flies learning has been studied in different sensory modalities – olfaction, vision, taste – and domains, such as foraging and social context.

The sensory modality that has been investigated in more detail in fruit flies is **olfaction**. This is due to the relative simplicity of this system (reviewed in Davis, 2004) and to the presence of interesting parallels of the olfactory nervous system between insects and vertebrates (reviewed in Davis, 2004). In olfactory learning studies conducted in the laboratory, flies were able to associate an odor with electric shocks (Tully and Quinn, 1985). They were also able to associate an odor with aversive or appetitive flavor (Mery and Kawecki, 2002). Olfactory learning has been also investigated in semi-natural environments. Flies free to fly in a greenhouse were able to associate an odor with appetitive or aversive taste (Zrelec et al., 2013).

Several studies have been conducted on **visual** learning as well. In a visual learning study, flies were able to remember visual patterns (Dill and Heisenberg, 1995). In this study flies were tied on a torque meter and four identical patterns were presented in the arena. During the second exposure a new and the previously presented pattern are presented to the fly. Flies preferred to fly towards the new pattern, which indicated that they remember the previously presented pattern. A similar method has been used also to assess flies ability in color vision (Wolf and Heisenberg, 1997). Flies learned to associate green or blue with heat, using a fly simulator, in which the fly was tied on a torque meter and the arena was heated up when either blue or green was present in the arena.

Researchers have started to investigate also **gustatory** learning in flies (Burke and Waddell, 2011). In this study flies learned to associate nutritious sugars with odor A and non-nutritious sugars with odor B. Flies were starved 16 to 20 hours before training and testing flies 24 hours after training in a T-maze had revealed that flies trained on nutritious sugars formed a robust memory, whereas non-nutritious sugars did not lead to memory formation.

Flies are also able to perform **auditory** learning: they were able to associate a sound with a sugar reward causing a PER even when no sugar was offered (Menda et al., 2011). Flies were also able to perform **motor** learning because they learned to keep the leg flexed to avoid an electric shock (Booker and Quinn, 1981).

Recently researchers started to focus on **social** learning in larvae and adult fruit flies. This system model can in fact be used to simplify the investigation of complex behaviors in a model organism easily handle. *D. melanogaster* larvae were able to associate high-quality food with the presence of other larvae (Durisko and Dukas, 2013). In this study single larvae had the choice to choose either higher-quality food occupied by other larvae or fresh lower-quality food without larvae. The tested larvae preferred to choose the food used by other larvae, which suggests that they are learning from others.

The behavior of flies plays a critical role during olfactory conditioning. Sitters harboring the *for^S* variant had a better learning performance when they were conditioned and tested with other flies compared to conditioning and testing alone. Rovers harboring the *for^R* variant didn't display any significantly change in learning performance when conditioned in a group or alone, which suggests that rovers are more independent of the social context than sitters (Kohn et al., 2013).

Summarizing, plenty of behavioral studies have shown that flies are able to learn in different sensory modalities, tasks and domains, showing the potential of this model system. A limitation of most of the procedures used to investigate learning in fruit flies is that they are very time consuming. The motor learning procedure for example requires much time because each tested fly has to be tied to a wire (Booker and Quinn, 1981). Furthermore many learning procedures are only suitable to condition and test individual flies or only a small group of flies or complex machines are necessary to condition flies (Tully and Quinn, 1985). Hence it is necessary to develop a method that overcomes these limitations.

In different learning (Mery and Kawecki, 2002) and perceptual (Dweck et al., 2013) experiments researchers noticed that fruit flies exhibit spontaneous preferences for particular odors. For instance, the control population and the flies selected for enhanced learning have a spontaneous preference for orange odor compared to pineapple odor. As recently discovered (Dweck et al., 2013) the citrus odor preference of *D. melanogaster* is likely to be a strategy evolved to avoid parasitic wasps, a natural *D. melanogaster* parasite that avoids citrus odor. Investigating the spontaneous preference for the experimental stimuli is essential, because this reveals how an unconditioned animal spontaneously chooses (reviewed in Gong, 2011). Having a spontaneous preference for one of the odors used in the tests can

bias the outcome of learning experiments. Estimating the spontaneous responses in the absence of conditioning is crucial to evaluate the effect of the conditioning procedure. For this reason any paradigm used for perceptual learning should be suitable to test spontaneous preferences too.

1.3 Research aims

My master project focused on different scientific aims: establishing a convenient behavioral method to phenotype learning and spontaneous preferences in fruit flies, investigate learning and perceptual preferences of a large population of fruit flies and investigate learning and perceptual preferences in inbred lines.

In the first part of my thesis project I focused on the establishment and testing of a new method, that enables researchers to condition and phenotype simultaneously a large group of flies from a natural population. With this new method I planned to save time and resources by testing large groups of flies with low effort. The method meant to be easy, maintenance-free and cheap and to be sensitive enough to detect learning and spontaneous preferences in a population not exposed to selection. Furthermore the new method was designed to avoid selection for females or for egg washing resistance, as the oviposition paradigm (Mery and Kawecki, 2002). Another aim was to test whether conditioned flies improve their performance after conditioning for more than one day. Using this method it should be possible to study different sensory modalities and domains, for example evaluating the spontaneous preference of different odors, the role of order presentation during the procedure and even studying the visual learning performance and spontaneous preference for different colors in *D. melanogaster*.

In the second part of my thesis project I focus on the study of genetic differences in the olfactory learning performance by using the behavioral paradigm previously validated on different inbred lines of *D. melanogaster*. Each inbred line has a different genetic background compared to the other lines but is raised and tested under identical conditions. Hence comparing the performance of different lines it is possible to investigate whether genetic differences among the lines affect their learning performance.

2 Materials and methods

In this section I first describe how *Drosophila* stocks of the South African *Drosophila melanogaster* population and the Portuguese *D. melanogaster* inbred flies were derived and maintained. Then, I describe the apparatus used for the olfactory learning paradigms, the olfactory learning procedures and the spontaneous preference paradigms. Finally, I mention the recipe for cooking experimental fly food, the statistical analyses performed and the software used for the statistical analyses.

2.1 *Drosophila* stocks

2.1.1 South African *D. melanogaster* isofemale lines

I used flies of 670 isofemale lines derived from a natural population of *Drosophila melanogaster* collected in Paarl (South Africa) in March 2012. Flies have been maintained on standard cornmeal-soy flour-syrup-yeast medium. Before starting the experiments I maintained the isofemale lines at 22°C in a constant 14:10 h light:dark cycle for at least two generations. At this temperature flies have a generation time of three weeks. During all experiments I used adult flies with an age of minimum one day from eclosion and a maximum age of 14 days from eclosion.

2.1.2 Portuguese inbred *D. melanogaster* lines

An population of *D. melanogaster* has been collected in Terroso and Pavoa (Portugal) in July 2008 and maintained as isofemale lines. From these isofemale lines eleven inbred lines assayed were generated through full-sibling mating. For each line a virgin female and a randomly collected male were allowed to mate and from their offspring another virgin female and a random male were used to create the next generation. Lines derived from the base stock population B101, B192 and B211 were maintained as isofemale lines in room temperature before they were inbred for 17 to 19 generations. Lines derived from replicates in the hot (constant 25°C, 12:12 h light:dark cycle) treatment R1, R2, R3 and R5 were maintained for 53 generations in the hot cage, before they were inbred for additional 21 to 29 generations. Lines obtained from replicates in the cold (constant 18°C, 12:12 h light:dark cycle) treatment R6, R7, R9 and R10 were maintained for 33 generations in the cold cage,

before they were inbred for additional 20 to 22 generations. The flies were maintained on standard cornmeal-soy flour-syrup-yeast medium. Before the beginning of the experiments I maintained all lines for at least two generations at 22°C in a constant 14:10 h light:dark cycle. All flies used during experiments were at least one day old (counted from hatching). The exact generation number of inbreeding for each line is shown in Table 2.1.

Table 2.1 Generation number for inbred *D. melanogaster* lines provided from the Institute of Population Genetics in Vienna.

Line	R1	R2	R3	R5	R6	R7	R9	R10	B101	B192	B211
Generations of inbreeding	27	29	27	21	20	22	20	22	17	18	19

2.2 Olfactory phenotyping

2.2.1 Apparatus for olfactory phenotyping

The T-maze (31 x 17.5 cm) used for the olfactory phenotyping (Figure 2.1) consists of a transparent central T-chamber (12 x 8 x 1.5 cm) and two side chambers (9.5 x 2.5 x 2.5). At the entrance of the T-maze a standard *Drosophila* vial (9.5 x 2.5 cm) with starved flies can be connected using a sponge. On both ends of the T-maze a standard *Drosophila* vial filled with 4 ml of orange or apple juice medium is located. At the beginning of each subsequent experimental phase I supplied fresh media to the apparatus by using vials with fresh food. I connected the central chamber and the vials containing fly food with a narrow funnel. In this way flies were able to enter the fly food vials, but once inside could not escape. To control for equal lightening, I placed neon lamps (Ultra Slim T4/20W/G5 with 50 Hz) at 41 cm distance on top/center of each apparatus.
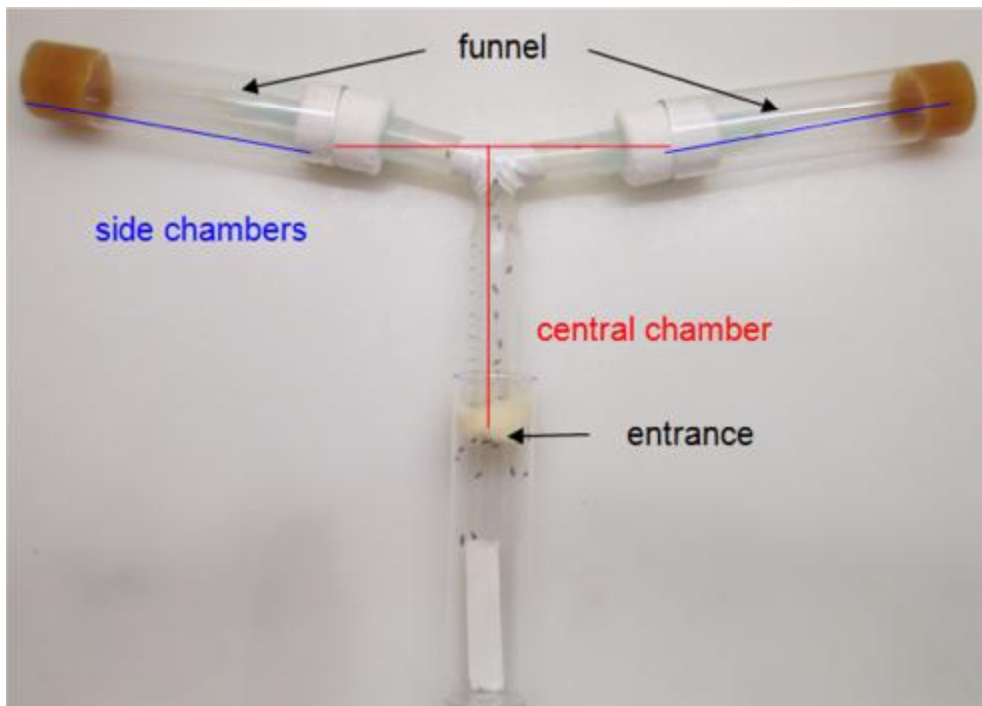
Figure 2.1. Apparatus for olfactory phenotyping.
Image of the T-maze used during olfactory experiments. Orange or apple juice medium is supplied on both ends of the T-maze representing the side chambers. Starved flies can smell the odor associated to the fly food through the T-maze and once entered a vial and reached the food, they are "trapped" through a narrow funnel that makes the way out extremely difficult.

2.2.2 Olfactory learning experimental setup for the South African *D. melanogaster* population

Day 1

In this experiment flies were conditioned to associate apple or orange odor with either aversive or appetitive flavor through a repeated exposure procedure (Exposure 1 and Exposure 2). In Exposure 1 flies were exposed to either orange or apple odor added with quinine and in Exposure 2 flies were exposed to appetitive food previously not associated with aversive taste.

In each experimental trial I used a group of 250 flies (50:50 sex ratio), randomly collected from the 670 isofemale lines of the South African population. I used CO_2 anesthesia to collect them. Before the beginning of the experimental trials flies were starved. During starvation I kept flies for 15 hours in a standard *Drosophila* vial provided with moistened Whatman paper to prevent desiccation stress. Immediately

before the start of the experimental trials I provided fresh food to the apparatus. Then I connected the vial with the starved flies to the T-maze (Figure 2.1).

Exposure 1 lasted 2 hours. In Exposure 1 half of the trials were run adding quinine to apple juice medium and half were run adding quinine to orange juice medium. After two hours I collected flies trapped in the vials containing aversive food and transferred them in a separate empty vial. I discarded flies staying in the central chamber during this exposure phase because these flies have not been exposed to aversive food.

Exposure 2 immediately followed Exposure 1. Exposure 2 lasted 2 hours. In Exposure 2 I released previously collected flies in the T-maze, provided with vials containing appetitive food previously not associated with aversive flavor. After two hours I collected flies trapped in the vials containing appetitive food and starved them for four additional hours in a vial provided with moistened Whatman paper before testing them.

The Test phase lasted 1 hour. During the test, the T-maze was supplied on one side with food previously associated with aversive taste and on the other side with food previously associated with appetitive taste. In half of the runs the food previously associated with appetitive flavor was supplied on the right side of the apparatus, and in half of the runs, it was supplied on the left side of the apparatus. In the Test phase I used only medium without quinine. Every 30 minutes I collected flies trapped in the vials during the test. Then I counted how many flies chose the odor previously associated with appetitive or aversive taste.

Day 2

For the second day I collected and pooled flies trapped in aversive and appetitive food during the test phase of Day 1 and starved them for 15 hours. Then I repeated the whole experimental procedure with the pooled flies in Day 2, to test whether flies improved their learning performance after a subsequent conditioning day (see 2.2.2 Day 1).

2.2.3 Olfactory learning experimental setup for Portuguese *D. melanogaster* inbred lines

To investigate the olfactory learning performance of the inbred lines, I used 40 flies (50:50 sex ratio), collected from each inbred line separately. I used the same procedure described in the olfactory learning experimental setup for the South African *D. melanogaster* population (Figure 2.2.), except that the Test phase lasts 1.5 hours and I conditioned flies only one day. During the test I collected flies after 30 minutes and then after 1 hour.

n = 250 or 40 flies

15 h starvation

(a) Exposure 1 phase (2 h)

apple odor

funnel to trap flies

apple juice with quinine

(b) Exposure 2 phase (2 h)

funnel to trap flies

orange odor

orange juice

4 h starvation

(c) Test (1 h)

orange odor

orange juice

apple odor

apple juice
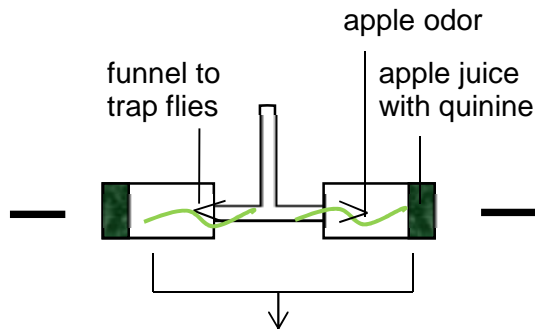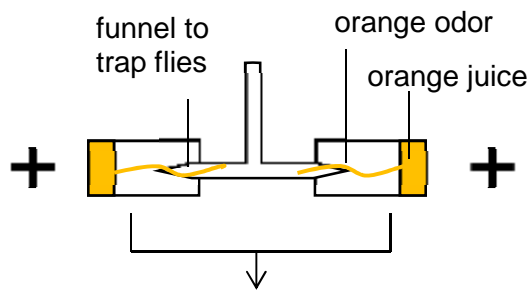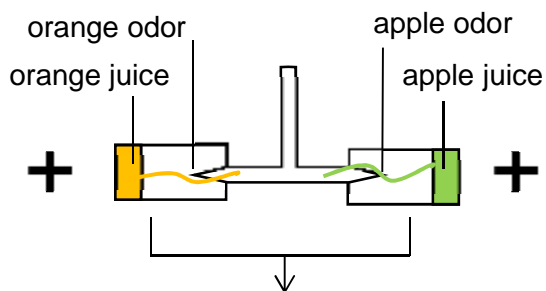
collect good and poor learners and repeat assay next day

Figure 2.2. The olfactory learning experimental setup.
Starved flies can learn to associate an unconditioned stimulus (e.g. aversive (-) or pleasant (+) food) with the associated cue (e.g. apple or orange odor). In Exposure 1 (a) the unconditioned aversive food (e.g. apple juice supplemented with quinine) is associated with a cue (e.g. apple odor). During Exposure 2 (b) the unconditioned appetitive stimulus is associated with another cue (e.g. orange odor). After four hours of starvation flies are tested (c) offering them both odors without quinine. After the test the number of flies that chose the odor previously associated with appetitive or aversive flavor is measured.

2.2.4 Avoidance rate assay

As preliminary assay, I tested if 8 g/L quinine leaded to an avoidance rate of 90 % for aversive food. This avoidance rate has been used by Mery and Kawecki (2002) and Zrelec et al. (2013) in previous olfactory learning assays in fruit flies. I released a group of 200 flies (50:50 sex ratio) derived from the South African population in a cage (39 x 28 x 28 cm) containing two bottles (6 oz *Drosophila* stock bottle) that were placed in a central position at a distance of seven centimeters. One bottle contained appetitive orange juice medium and the other one aversive orange juice medium (supplemented with 8g/L quinine). During 24 hours flies had the choice to oviposit on both media. Then I counted eggs laid in both food types and calculated the avoidance rate. Since this quinine concentration produced a 90 % avoidance rate, I used the 8g/L quinine concentration for the aversive food supplied during Exposure 1 in the olfactory learning experimental setup.

2.2.5 Spontaneous odor preference assay for South African *D. melanogaster* population

In this experiment I measured the spontaneous preference for the two experimental stimuli (orange and apple juice medium) without prior conditioning. In each experimental trials I used a group of 250 flies (50:50 sex ratio), randomly collected from the 670 isofemale lines of the South African *D. melanogaster* population and treated them in a similar way as described in the olfactory learning setup (Figure 2.3). The only difference to the previously explained procedure was the absence of quinine during Exposure 1 phase: in this way the learning assays are comparable to the spontaneous preference assays.

2.2.6 Spontaneous odor preference assay for Portuguese *D. melanogaster* inbred lines

To determine the spontaneous preference of the experimental stimuli (orange and apple juice medium) for the inbred lines, I used 40 flies (50:50 sex ratio), from each inbred line separately and treated them the same way as the South African *D. melanogaster* population (Figure 2.3).

n = 250 or 40 flies

15 h starvation

(a) **Exposure 1 phase (2 h)**

(b) **Exposure 2 phase (2 h)**

4 h starvation

(c) Preference Test (1 h)

collect orange and apple choosers and repeat assay next day
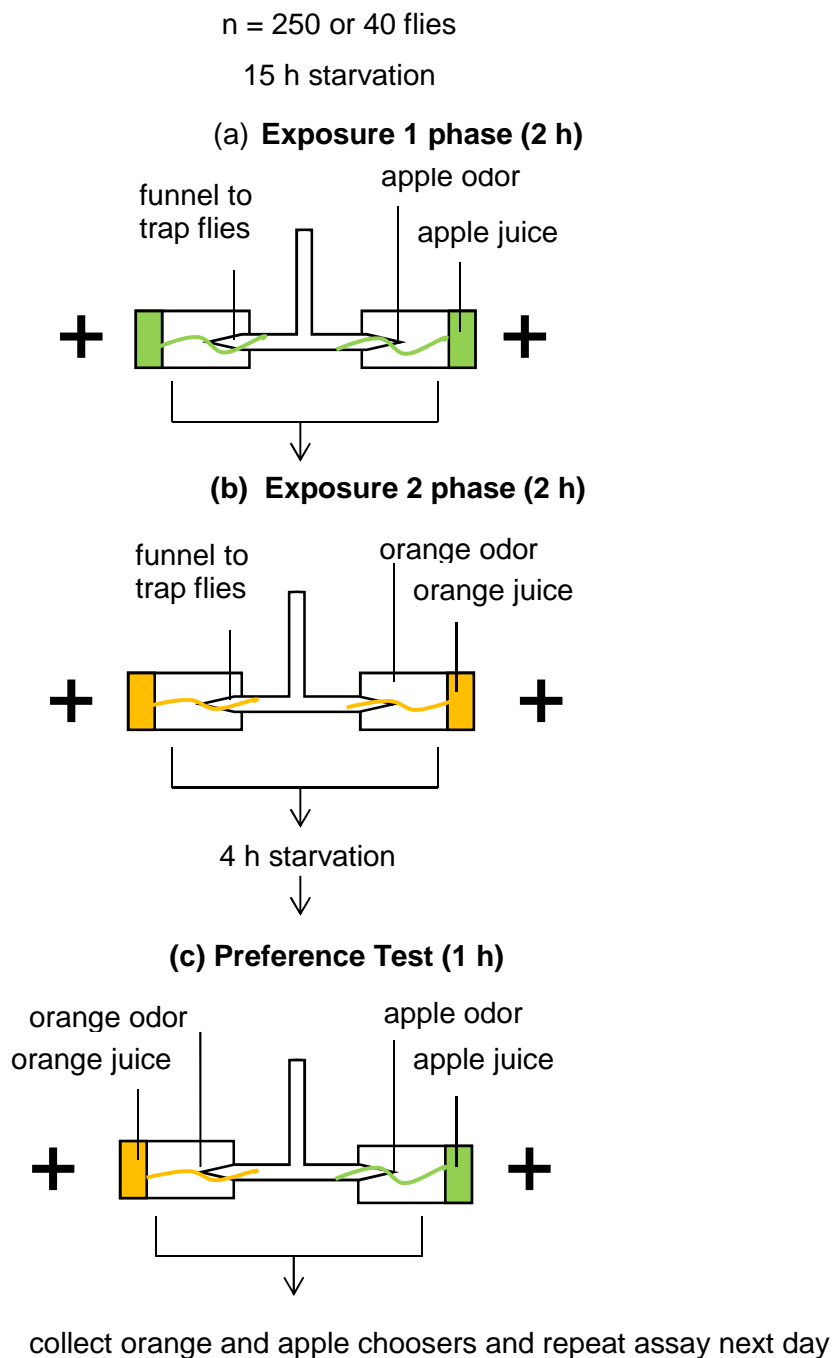
Figure 2.3. The spontaneous odor preference assay.
Starved flies were exposed to appetitive (+) food with different odors. In Exposure 1 (a) appetitive food (e.g. apple juice) with its associated cue (e.g. apple odor) was offered. In Exposure 2 (b) appetitive food (e.g. orange juice) with its associated cue (e.g. orange odor) was presented. After four hours of starvation flies were tested (c) offering them both odors. After the Test phase the number of flies that chose orange or apple odor was measured.

2.3 Fly food recipes

2.3.1 Fly food used in the phenotyping assays

Fly food with orange and apple flavor used during the olfactory experiments was cooked following the recipe reported in Table 2.1.

Table 2.2 Recipe for flyfood used during olfactory experiments.

Appetitive medium		Aversive medium	
0.5 L	orange or apple juice from concentrate	0.5 L	orange or apple juice from concentrate
+ 7 g	agar-agar	+ 7 g	agar-agar
+ 60 ml	malt syrup	+ 60 ml	malt syrup
	bring to boil		bring to boil
+ 1 g + 2.5 ml	nipagine solubilized in ethanol	+ 1 g + 2.5 ml	nipagine solubilized in ethanol
+ 2 ml	propionic acid	+ 2 ml	propionic acid
		+ 4 g	quinine hydrochloride
	stir and pour into vials		stir and pour into vials

2.4 Data analysis

To analyze the performance of fruit flies in each experimental trial I first calculated the proportion of flies that chose the orange odor (orange choosers) in different experiments and conditions.

Phenotyping assays. For flies conditioned to choose orange odor I expected flies that could remember the association between apple odor and the aversive food, increased their probability to choose orange odor compared to apple odor (1) during the test after conditioning. For flies conditioned to choose the apple odor I expected a decrease in the proportion of orange odor compared to apple odor choosers after conditioning. By comparing the proportion of orange odor choosers in the group of flies conditioned to choose orange odor and conditioned to choose apple odor, it is possible to identify whether the conditioning procedure increases the probability of orange odor choices in flies conditioned to choose orange odor compared to flies conditioned to choose apple odor.

$$(1)\ \text{Orange choosers} = \frac{\text{Flies that choose orange odor}}{\text{Overall number of flies that choose orange or apple odor}}$$

2.5 Statistical tools

For data analysis and plots I used the open access software R version 2.12.1.

3 Results

3.1 South African *D. melanogaster* population results

In this section I describe the results of the olfactory learning experiment and the spontaneous olfactory preferences in the *D. melanogaster* population derived from a natural South African population.

3.1.1 Olfactory learning in the South African *D. melanogaster* population

I investigated the olfactory learning performance of this population after conditioning flies to associate either apple or orange odor with aversive or appetitive flavor. After conditioning I measured the proportion of orange odor choosers for flies conditioned to choose orange or apple odor.

In the presence of a learning effect induced by the conditioning procedure I expected significantly more orange odor choosers (flies that choose orange odor) in the samples conditioned to choose orange than in the samples conditioned to choose apple.

$$\text{Orange choosers} = \frac{\text{Flies that choose orange odor}}{\text{Overall number of flies that choose orange or apple odor}}$$

To investigate whether the learning performance improves after additional experience, the conditioning procedure was repeated for two consecutive days. I expected that the learning performance improved after the second day of conditioning, producing an increase in the learning score from day 1 to day 2.

3.1.1.1 Learning performance in day 1

Figure 3.1A shows the proportion of orange odor choices after conditioning in day 1 for flies conditioned to choose orange odor and flies conditioned to choose apple odor tested in 28 trials. The Shapiro-Wilk test showed that the data for flies conditioned to choose orange odor (W = 0.955, p = 0.251) and flies conditioned to choose apple odor (W = 0.952, p = 0.205) were not significantly different from a normal distribution (Table 3.1). Thus it was appropriate to use the Two Sample *t* test as a significance test, to test whether the proportion of orange choosers was significantly different between the two different conditioning regimes. If flies conditioned to choose orange odor had a significantly higher proportion of orange choices than flies conditioned to choose apple odor I could use this evidence to conclude that flies learned as a consequence of the applied conditioning regime. Flies conditioned to choose orange odor made significantly more choices for orange odor compared to flies conditioned to choose apple odor (t = 2.237, df = 55.755, p-value = 0.029) (Table 3.1). These results show that **flies learned to associate an odor with appetitive or aversive taste and that they were also able to form a memory trace and recall this memory during the test. The adopted procedure provides an effective method to condition large groups of flies and test the learning capabilities of flies in the absence of selection for enhanced learning**.

Figure 3.1. Proportion of orange odor choosers of conditioned South African *D. melanogaster* during day 1.
The yellow bar (Mean = 0.54, SE = 0.03) indicates the proportion of orange odor choices for flies conditioned to choose apple odor and the orange bar (Mean = 0.63, SE = 0.03) displays the proportion of orange odor choices for flies conditioned to choose orange odor.

3.1.1.2 Learning performance in day 2

If the proportion of orange choosers for flies conditioned to choose orange odor was significantly higher compared to flies conditioned to choose apple odor, this would indicate that flies learn to associate an odor with aversive or appetitive taste and that they were able to form and recall memory regarding the recently acquired new information. The Shapiro-Wilk test showed that the data for flies conditioned to choose orange odor ($W = 0.977$, $p = 0.777$) were not significantly different from a normal distribution but the data for flies conditioned to choose apple odor ($W = 0.910$, $p = 0.019$) were not normally distributed, thus I applied a non-parametric test (Table 3.1). In spite of the higher number of orange odor choices of flies conditioned to choose orange odor, flies conditioned to choose orange odor made not significantly more choices for orange odor compared to flies conditioned to choose apple during the second day ($U = 471.5$, $p = 0.195$) (Figure 3.2, Table 3.1).

Although I expected flies conditioned for two subsequent days to have a significant effect of learning and to improve their learning performance compared to flies conditioned for a single day, flies did not statistically improve their learning performance after a consecutive conditioning. In spite of this I observed for the day 2 a trend similar to day 1, namely increasing proportion of flies choosing the odor previously associated with appetitive food. The discrepancy between expected and observed results could be due to a decrease of statistical power from day 1 to day 2 due to a smaller starting sample size (after starvation) in day 2 (Mean = 74.75, SE = 4.472) compared to day 1 (Mean = 232.31, SE = 2.554) and a reduced number of choosers from day 1 (Mean = 96.03, SE = 5.44) to day 2 (Mean = 52.71, SE = 5.08). Thus to increase statistical power it would be necessary to start with a larger starting sample in day 1 to obtain a bigger starting sample in day 2. Given that there is an average loss of 68 % of subjects between day 1 and day 2 and an additional 10 % loss of choosers from day 1 to day 2, **to guarantee a sufficient number of choosers (about 100) in day 2 one should increase the starting population of day 1 to at least 425 flies**.
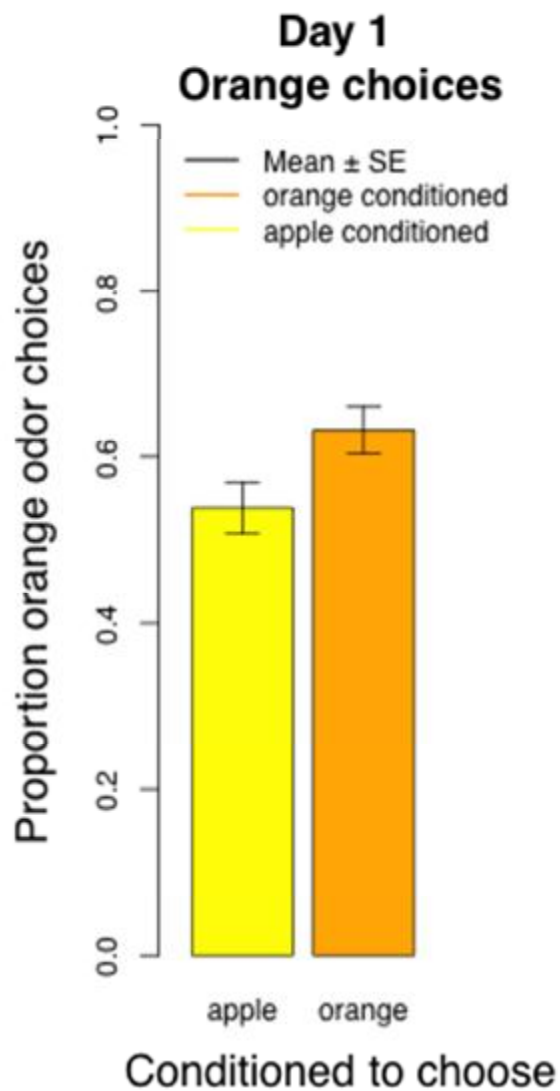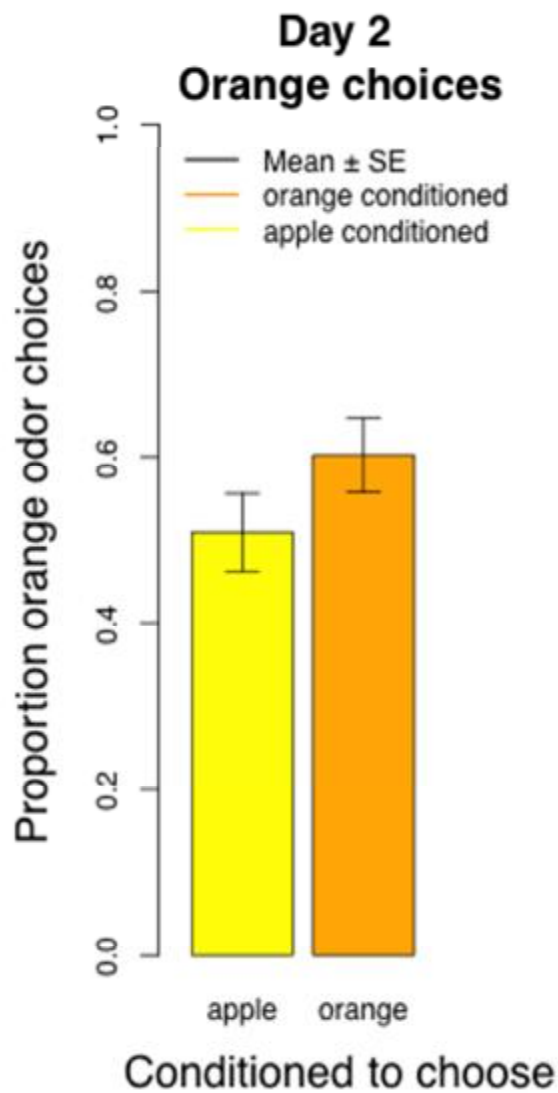
Figure 3.2. Proportion of orange odor choosers of conditioned South African *D. melanogaster* during day 2.
The yellow bar (Mean = 0.51, SE = 0.05) represents the proportion of orange odor choices for flies conditioned to choose apple odor and the orange bar (Mean = 0.60, SE = 0.04) indicates the proportion of orange odor choices for flies conditioned to choose orange odor.

Table 3.1 Data analysis of the olfactory learning performance in day 1 and day 2.

Analysis and tests	Day 1		Analysis and tests	Day 2	
Distribution analysis:	*Conditioned to choose orange odor*	*Conditioned to choose apple odor*	**Distribution analysis:**	*Conditioned to choose orange odor*	*Conditioned to choose apple odor*
Shapiro-Wilk test	W = 0.955 p = 0.251	W = 0.952 p = 0.205	**Shapiro-Wilk test**	W = 0.977 p = 0.777	W = 0.910 p= 0.019
Significance analysis:	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor*		**Significance analysis:**	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor*	
Two Sample *t* test (Welch correction)	t = 2.237 df = 55.755 p = 0.029		**Wilcoxon rank-sum test**	*U* = 471.5 p = 0.195	

3.1.2 Spontaneous preference for orange and apple odor in the South African *D. melanogaster* population

I investigated the spontaneous preference for orange and apple odor in the South African *D. melanogaster* population in the two consecutive days of test (day 1 and day 2). A significant preference for one of the two odors used in the test would imply the capability to discriminate between them.

$$\text{Orange preference} \quad = \quad \frac{\text{Flies that choose orange odor}}{\text{Overall number of flies that choose orange or apple odor}}$$

As I observed that flies exhibit a different learning performance depending on the order of exposure in the two conditioning procedures, I also studied whether the order of odor presentation makes a significant difference in the spontaneous preference of naïve flies.

3.1.2.1 Spontaneous odor preference in day 1

I investigated if unconditioned flies exhibit a preference for orange odor without dividing by the order of odor presentation during day 1 tested in 14 trials, comparing the proportion of orange odor choices to the chance level (Figure 3.3A). The Shapiro-Wilk test revealed that the data collected for both orders of presentation were not significantly different from a normal distribution (W = 0.971, p = 0.598) (Table 3.2). The One Sample t test showed that flies had a performance of orange odor choosers not significantly different from the chance level (t = 1.953, df = 27, p = 0.061) with a trend towards an overall preference for orange odor. Thus **the overall population exhibited a trend towards orange odor during day 1.**

To test whether the order of presentation had an effect on the spontaneous olfactory preference in day 1 I compared the proportion of orange odor choices for flies first exposed to apple and then to orange odor (A/O) and for flies first exposed to orange and then to apple odor (O/A) (Figure 3.3B and Table 3.2). A difference in the proportion of orange odor choosers would indicate that the olfactory preference can be affected by the order of odor presentation. The Shapiro-Wilk test showed that the data collected with the O/A order (W = 0.975, p = 0.936) and the A/O order (W = 0.927, p = 0.272) were not significantly different from a normal distribution. The One Sample t test revealed that flies assayed with the O/A order (t = -0.242, df = 13, p = 0.813) had a performance not significantly different from the chance level, but with the A/O order flies' preferences were significantly different from the chance level (t = 3.185, df = 13, p = 0.007). Furthermore the Two Sample t test revealed a significantly higher proportion of orange odor choosers for the A/O order compared to the O/A order (t = 2.464, df = 25.96, p = 0.021). Summarizing **flies preferred to choose orange odor when exposed to A/O but not when exposed to the O/A order: this shows that the order of presentation affects the olfactory preferences of unconditioned flies.**

Figure 3.3. Spontaneous odor preference of the South African *D. melanogaster* population and the resulting order score during day 1.
A The gold bar (Mean = 0.52, SE = 0.01) displays the proportion of orange odor choices without dividing by the order of odor presentation (A/O + O/A).
B The orange bar (Mean = 0.55, SE = 0.02) indicates the proportion of orange odor choices of flies first exposed to apple and then to orange odor (A/O). The yellow bar (Mean = 0.50, SE = 0.02) represents the proportion of orange odor choices of flies first exposed to orange and then to apple odor (O/A).

3.1.2.2 Spontaneous odor preference in day 2

I investigated if unconditioned flies exhibit a preference for orange odor without considering the order of odor presentation after one and two exposure days tested in 14 trials, comparing the proportion of orange odor choices (Figure 3.4A). I expected to observe a similar spontaneous preference in the first and the second day. The Shapiro-Wilk test revealed that the data collected for both orders of presentation were not significantly different from a normal distribution (W = 0.960, p = 0.339) (Table 3.2). The One Sample *t* test showed that flies exhibit a performance of orange odor choosers significantly higher compared to the chance level (t =3.484, df = 27, p = 0.002). Thus **flies exhibit a general preference for orange odor without dividing by the order of odor presentation during day 2.** This result suggests that the preference for the orange odor present in day 1 increased in day 2.

Then I studied if unconditioned flies exhibit a different preference for orange odor when exposed to different orders of odor presentation in day 2. In Figure 3.4B I compared the proportion of orange odor choosers between flies first exposed to apple odor (A/O) and flies first exposed to orange odor (O/A). I used a normality test to analyze the distribution of the data (Table 3.2). The Shapiro-Wilk test revealed that the data collected with the O/A order (W = 0.815, p = 0.008) were significantly different from a normal distribution, but the data for the A/O order (W = 0.898, p = 0.104) were not significantly different from a normal distribution. The Wilcoxon rank-sum test revealed that flies assayed with the A/O order had a performance not significantly different from the chance level (V = 58, p = 0.761) whereas flies' orange odor preferences were significantly higher compared to the chance level with the O/A order (V = 105, p = 0.0001). Furthermore the Wilcoxon rank-sum test revealed that the proportion of orange odor choosers for the A/O order was significantly lower compared to the O/A order (W = 38, p = 0.005).

Summarizing, flies significantly preferred to choose orange odor when exposed to O/A compared to the A/O order, this showed that the order of presentation affects the spontaneous preference of unconditioned flies also during an additional exposure day. Orange odor choices were only significantly higher compared to the chance level when flies were exposed to O/A.

I expected the spontaneous preference of the second day to be similar to the performance of the first exposure day. Interestingly **flies increased their preference for orange odor after two days of exposure.**
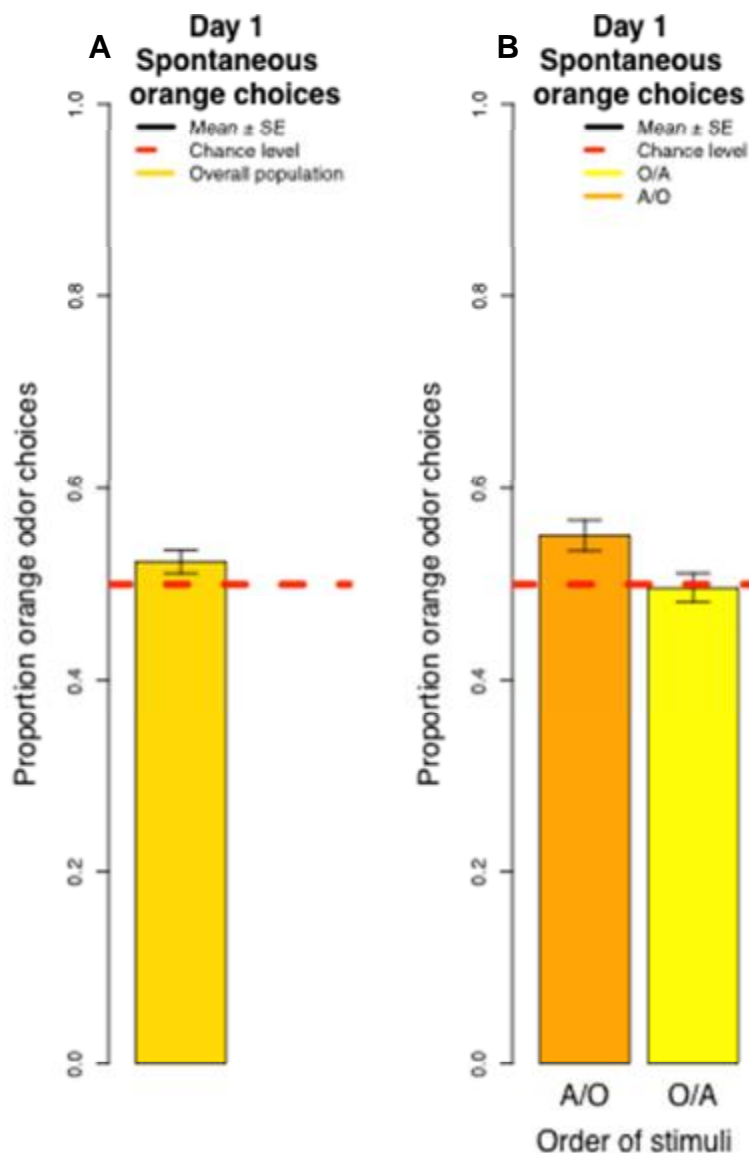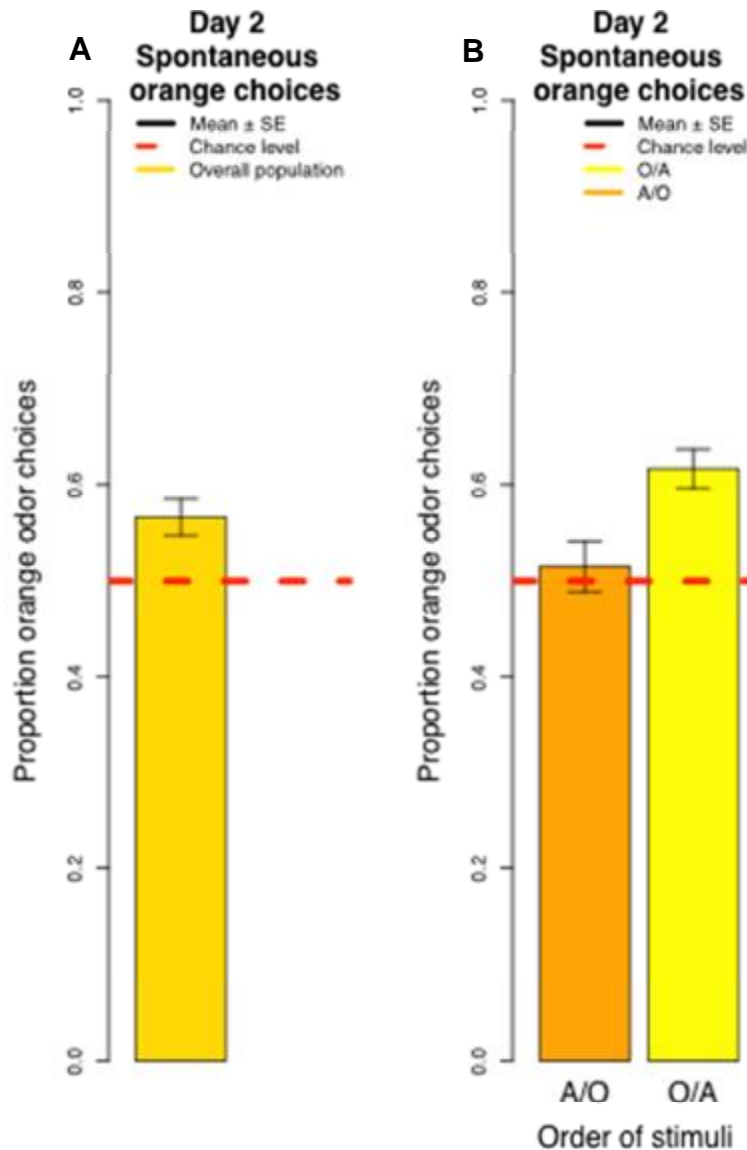

Figure 3.4. Spontaneous odor preference of the South African *D. melanogaster* population and the resulting order score during day 2.
A The gold bar (Mean = 0.57, SE = 0.019) displays the proportion of orange odor choices without dividing by the order of odor presentation (A/O + O/A).
B The orange bar (Mean = 0.52, SE = 0.03) indicates the proportion of orange odor choices of flies first exposed to apple and then to orange odor (A/O). The yellow bar (Mean = 0.62, SE = 0.02) represents the proportion of orange odor choices of flies first exposed to orange and then to apple odor (O/A).

Table 3.2. Statistical analysis of spontaneous odor preference in day 1 and day 2.

Analysis and tests	Day 1: spontaneous preference		Analysis and tests	Day 2: spontaneous preference	
Distribution analysis: **Shapiro-Wilk. test**	*without dividing by the order of presentation (A/O + O/A)* W = 0.971 p = 0.598		**Distribution analysis:** **Shapiro-Wilk. test**	*without dividing by the order of presentation (A/O + O/A)* W = 0.960 p = 0.339	
Significance analysis: One Sample *t* test	*A/O + O/A different from 0.5* t = 1.953 df = 27 p = 0.061		**Significance analysis: One Sample *t* test**	*A/O + O/A different from 0.5* t = 3.484 df = 27 p = 0.002	
Distribution analysis: Shapiro-Wilk test	*A/O* W = 0.927 p = 0.272	*O/A* W =0.975 p = 0.936	**Distribution analysis: Shapiro-Wilk test**	*A/O* W = 0.898 p = 0.104	*O/A* W = 0.815 p= 0.008
Significance analysis: **One Sample *t* test**	*A/O different from 0.5* t = 3.185 df = 13 p = 0.007	*O/A different from 0.5* t = -0.242 df = 13 p = 0.813	**Significance analysis:** **Wilcoxon signed-rank test for O/A**	*A/O different from 0.5* V = 58 p = 0.761	*O/A different from 0.5* V = 105 p = 0.0001
Significance analysis: Two Sample *t* test (Welch correction)	*A/O different from O/A* t = 2.464 df = 25.96 p = 0.021		**Significance analysis: Wilcoxon rank-sum test**	*A/O different from O/A* W = 38 p = 0.005	

3.2 Average numbers of conditioned flies for the South African population left at every step of the conditioning procedure

In Table 3.3 I present how many flies are left at every step during the conditioning procedure. Each experiment started with 250 flies per trial and 34 flies (13 %) died on average after 15 hours of starvation during the first day. Then I calculated how many flies made the right choice after the first (43) and the second test (9) in total 51 flies. Further I calculated how many flies made the wrong choice after the first (38) and the second (8) test in total 45 flies. This means that 96 flies made a choice during the first day. 25 % of these choosers died during the second starvation which means that 72 flies survived the starvation. The increase of the proportion of dead flies from day 1 (13 %) to day 2 (25 %) may be due to increased stress and consequently reduced fitness after this conditioning procedure. During the second day 27 flies in total made the right choice and 26 flies in total the wrong choice. This means that 53 flies chose during the second conditioning procedure. Interestingly chose more flies (53) during the second day in comparison to the number of starting flies (96) compared to the first day (250). This could be due to the fact that those choosers already knew the procedure and the construction of the T maze. Another possibility would be that the number of flies present in the T maze influences the choosing performance of flies. This would mean that the less flies present in the T maze the better the learning performance.

Steps of the conditioning procedure	Day 1	Day 2
Ø Number of start flies:	250	96
Ø Number of flies survived after starvation:	216	72
Ø Number of dead flies after starvation:	34	24
Proportion of dead flies after starvation:	**0.13**	**0.25**
Ø Number of flies making the right choice after the first test:	43	23
Ø Number of flies making the wrong choice after the first test:	38	23
Ø Number of flies making the right choice after the second test:	9	4
Ø Number of flies making the wrong choice after the second test:	8	4
Ø Sum of flies making the right choice:	51	27
Proportion of good learners:	**0.53**	**0.51**
Ø Sum of flies making the wrong choice:	45	26
Proportion of poor learners:	**0.47**	**0.49**
Total number of flies choosing during test 1 and test 2:	**96**	**53**

Table 3.3. Average numbers of conditioned flies left at every step of the conditioning procedure.

This table shows the average numbers of flies left at every step after the conditioning procedure and calculated proportions.

3.3 Portugal *D. melanogaster* inbred lines results

The results of the olfactory learning and the spontaneous odor preference of *D. melanogaster* inbred lines derived from a natural population in Portugal are described in this section. I tested eleven inbred lines (B101, B192, B211, R1, R2, R3, R5, R6, R7, R9 and R10). Line R6 consistently did not choose any odor during experiments, thus I excluded this line from the analysis.

3.3.1 Olfactory learning in the Portuguese *D. melanogaster* inbred lines

First I investigated if the overall inbred population exhibits a significant learning performance tested in 8 trials by analyzing the proportion of orange choosers for flies conditioned to choose orange or apple odor (Figure 3.5). The Shapiro-Wilk test revealed that the data for flies conditioned to choose orange odor ($W = 0.967$, $p = 0.034$) were not normally disturbed but the data for flies conditioned to choose apple odor ($W = 0.983$, $p = 0.382$) were not significantly different from normal distribution. The Wilcoxon signed-rank test revealed a significant difference for orange odor choices for flies conditioned to choose orange vs. apple odor for the overall population ($U = 4931.5$, $p = 3.46 \times 10^{-9}$) (Table 3.4). **This shows that the overall Portuguese *D. melanogaster* population significantly learned to associate an odor with appetitive or aversive taste.**

I analyzed if each inbred line separately was able to perform olfactory learning by analyzing the proportion of orange odor choices for flies conditioned to choose orange vs. apple odor (Figure 3.6). Only data from line R10 for flies conditioned to choose orange odor had a distribution significantly different from normality using the Shapiro-Wilk test ($W = 0.787$, $p = 0.021$) (Table 3.5). Thus I applied the Wilcoxon signed-rank test and the Bonferroni-Holmes correction for multiple comparisons for correction of the p-values. This analysis revealed that five out of ten lines significantly learned (R1: $p = 0.03$, R2: $p = 0.04$, R3: $p = 0.02$, R7: $p = 0.02$ and R9: $p = 0.002$) (Figure 3.6 and Table 3.5). **Thus these inbred lines were able to learn and associate an odor with appetitive or aversive taste and they could recall this memory trace. Hence the suggested method can be effectively used to condition small groups of flies in the absence of selection for enhanced learning.**
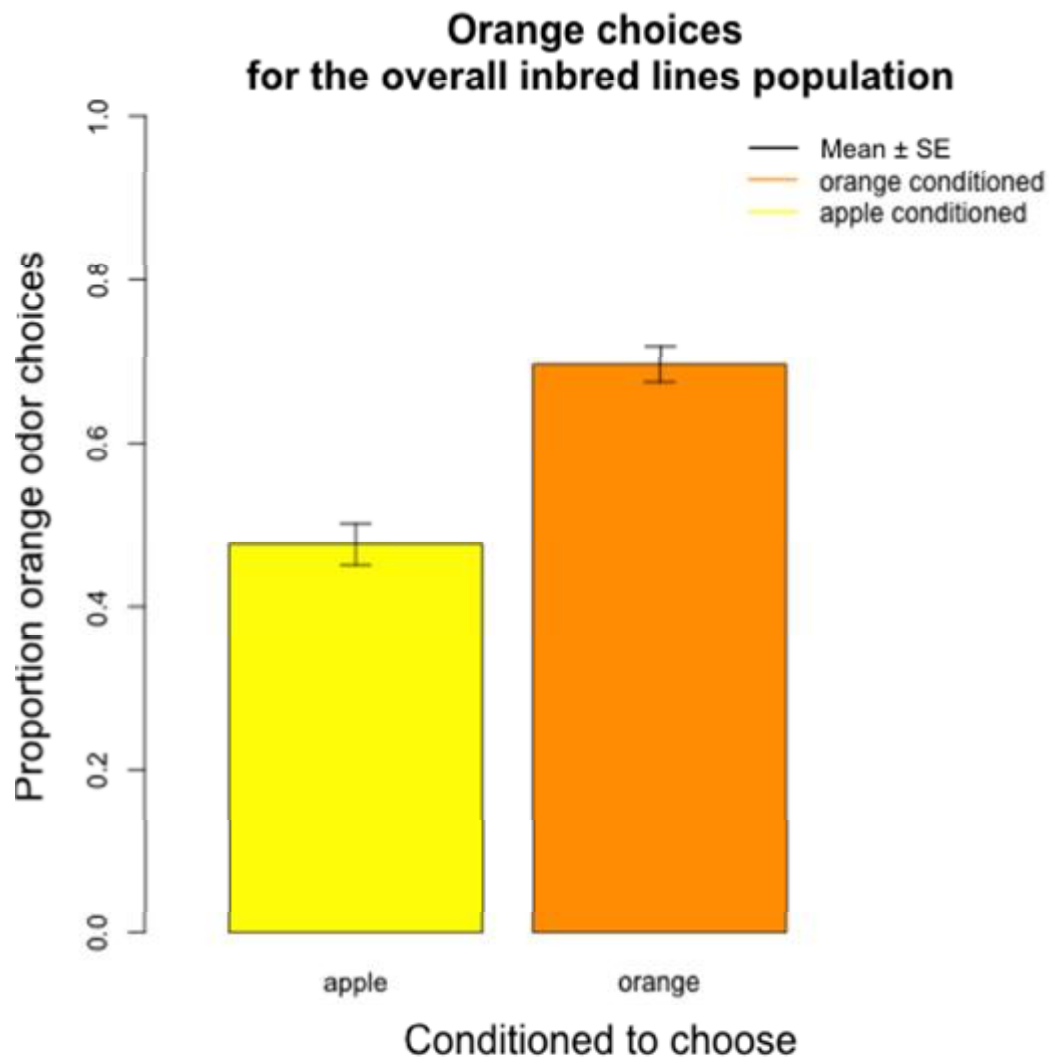
Figure 3.5. Proportion of orange odor choosers of the entire conditioned inbred *D. melanogaster* population.
The yellow bar (Mean = 0.48, SE = 0.03) shows the proportion of orange odor choices for flies conditioned to choose apple odor and the orange bar (Mean = 0.70, SE = 0.02) represents the proportion of orange odor choices for flies conditioned to choose orange odor.

Table 3.4. Data analysis of the olfactory learning performance for the entire inbred population.

Distribution analysis: Shapiro-Wilk test	*Conditioned to choose orange odor* W = 0.967 p = 0.034	*Conditioned to choose apple odor* W = 0.983 p = 0.382
Significance analysis: **Wilcoxon rank-sum test)**	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor* V = 4931.5 p = 3.46x10^{-9}	
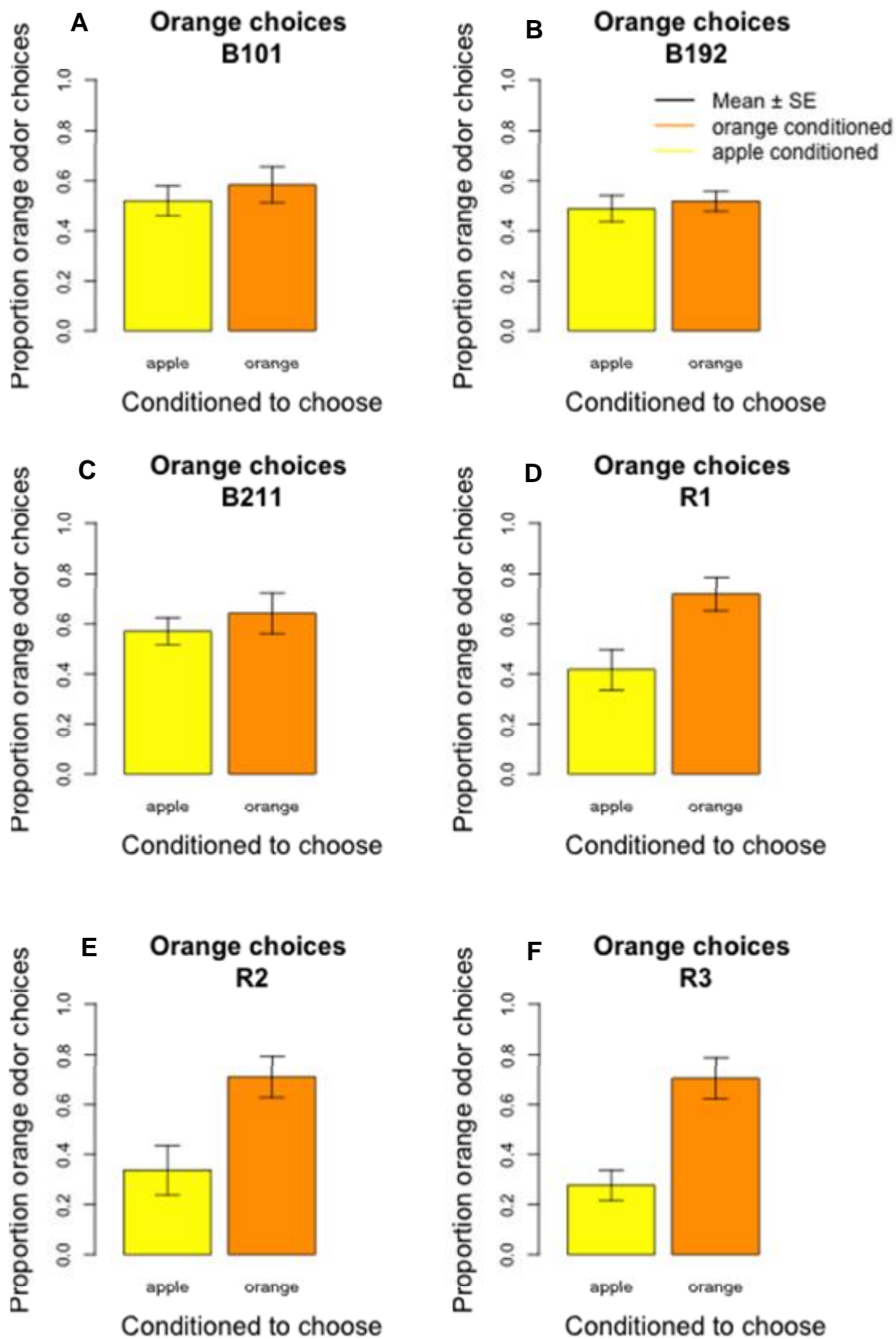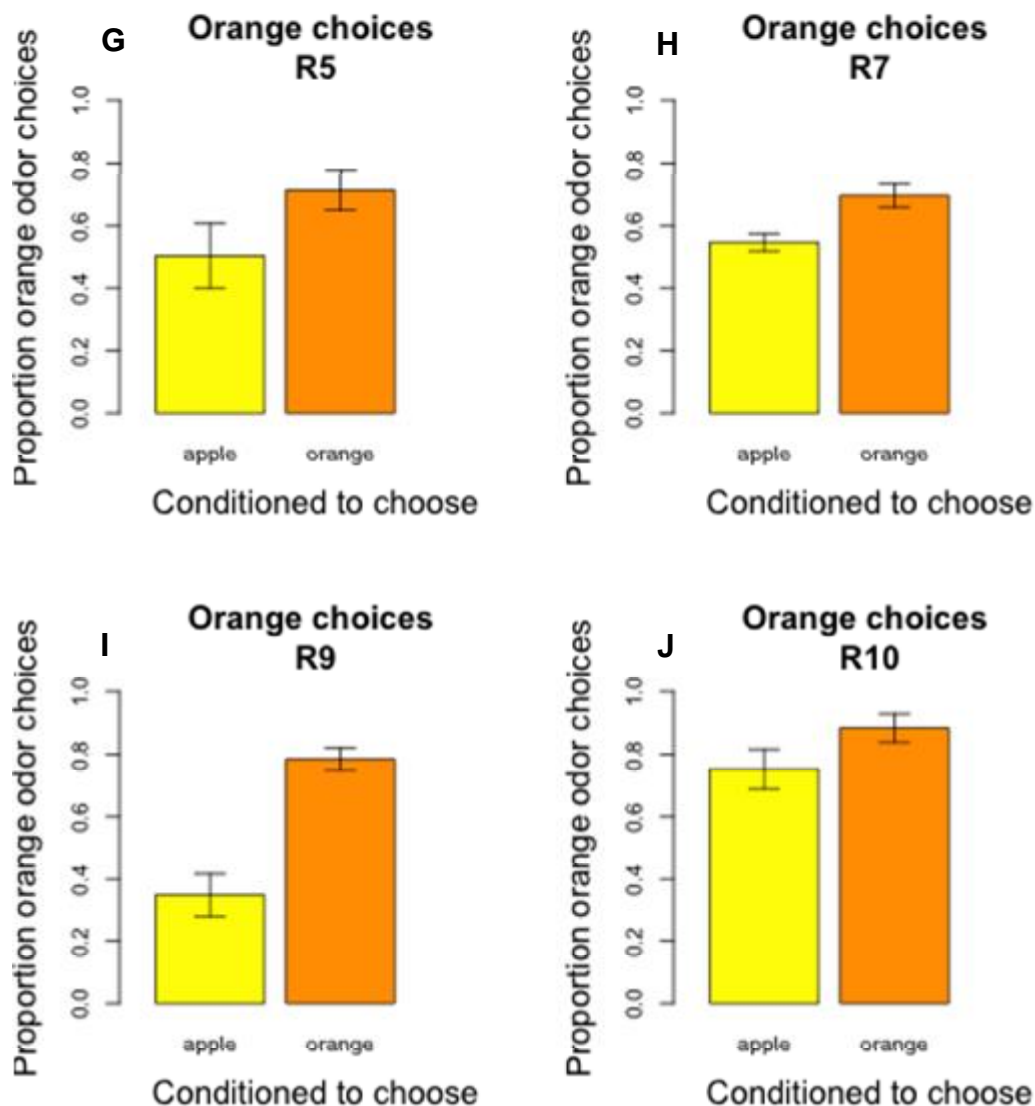
Figure 3.6. Proportion of orange odor choosers of conditioned inbred lines.
A The yellow bar (Mean = 0.52, SE = 0.06) shows the proportion of orange odor choices for B101 conditioned to choose apple odor and the orange bar (Mean = 0.58, SE = 0.07) represents the proportion of orange odor choices for B101 conditioned to choose orange odor.
B The yellow bar (Mean = 0.49, SE = 0.05) shows the proportion of orange odor choices for B192 conditioned to choose apple odor and the orange bar (Mean = 0.52, SE = 0.04) represents the proportion of orange odor choices for B192 conditioned to choose orange odor.
C The yellow bar (Mean = 0.57, SE = 0.05) shows the proportion of orange odor choices for B211 conditioned to choose apple odor and the orange bar (Mean = 0.64, SE = 0.08) represents the proportion of orange odor choices for B211 conditioned to choose orange odor.
D The yellow bar (Mean = 0.42, SE = 0.08) shows the proportion of orange odor choices for R1 conditioned to choose apple odor and the orange bar (Mean = 0.72, SE = 0.07) represents the proportion of orange odor choices for R1 conditioned to choose orange odor.

E The yellow bar (Mean = 0.34, SE = 0.10) shows the proportion of orange odor choices for R2 conditioned to choose apple odor and the orange bar (Mean = 0.71, SE = 0.08) represents the proportion of orange odor choices for R2 conditioned to choose orange odor.
F The yellow bar (Mean = 0.28, SE = 0.06) shows the proportion of orange odor choices for R3 conditioned to choose apple odor and the orange bar (Mean = 0.71, SE = 0.08) represents the proportion of orange odor choices for R3 conditioned to choose orange odor.
G The yellow bar (Mean = 0.50, SE = 0.10) shows the proportion of orange odor choices for R5 conditioned to choose apple odor and the orange bar (Mean = 0.71, SE = 0.06) represents the proportion of orange odor choices for R5 conditioned to choose orange odor.
H The yellow bar (Mean = 0.55, SE = 0.03) shows the proportion of orange odor choices for R7 conditioned to choose apple odor and the orange bar (Mean = 0.70, SE = 0.04) represents the proportion of orange odor choices for R7 conditioned to choose orange odor.
I The yellow bar (Mean = 0.35, SE = 0.07) shows the proportion of orange odor choices for R9 conditioned to choose apple odor and the orange bar (Mean = 0.78, SE = 0.04) represents the proportion of orange odor choices for R9 conditioned to choose orange odor.
J The yellow bar (Mean = 0.75, SE = 0.06) shows the proportion of orange odor choices for R10 conditioned to choose apple odor and the orange bar (Mean = 0.88, SE = 0.05) represents the proportion of orange odor choices for R10 conditioned to choose orange odor.

Table 3.5. Data analysis of the olfactory learning performance for each inbred line.

Inbred lines	Shapiro-Wilk test	Shapiro-Wilk test	Two Sample *t* test (Welch correction)	Wilcoxon signed-rank test	Wilcoxon test after Bonferroni-Holmes correction
	Flies conditioned to choose orange odor	*Flies conditioned to choose apple odor*	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor*	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor*	*Difference between flies conditioned to choose orange odor and conditioned to choose apple odor*
B101	W = 0.842 p = 0.079	W = 0.964 p = 0.851	t = 0.695 df = 13.63 p = 0.499	V = 42 p = 0.32	p = 0.35
B192	W = 0.936 p = 0.574	W = 0.914 p = 0.381	t = 0.453 df = 12.924 p = 0.658	V = 35 p =0.79	p = 0.79
B211	W = 0.858 p = 0.115	W = 0.911 p = 0.358	t = 0.736 df = 12.109 p = 0.476	V = 44.5 p =0.21	p = 0.29
R1	W = 0.964 p = 0.849	W = 0.969 p = 0.891	t = 2.886 df = 13.417 p = 0.012	V = 56.5 p =0.01	p = 0.029
R2	W = 0.923 p = 0.453	W = 0.914 p = 0.385	t = 2.94 df = 13.653 p = 0.011	V = 54 p =0.02	p = 0.04
R3	W = 0.94 p = 0.611	W = 0.931 p = 0.526	t = 4.216 df = 12.743 p = 0.001	V = 60 p =0.004	p = 0.02
R5	W = 0.901 p = 0.297	W = 0.895 p = 0.262	t = 1.724 df = 11.531 p = 0.111	V = 44 p =0.23	p = 0.29
R7	W = 0.96 p = 0.810	W = 0.875 p = 0.168	t = 3.226 df = 12.942 p = 0.007	V = 57 p =0.007	p = 0.02
R9	W = 0.869 p = 0.147	W = 0.948 p = 0.687	t = 5.64 df = 10.584 p = 0.0002	V = 64 p =0.0002	p = 0.002
R10	W = 0.787 p = 0.021	W = 0.955 p = 0.760	t = 1.682 df = 12.634 p = 0.117	V = 47 p =0.12	p = 0.20

3.3.2 Spontaneous odor preference for Portuguese *D. melanogaster* inbred lines

In Figure 3.7 I presented the proportion of orange odor choosers of the overall unconditioned population without dividing by the order of odor presentation tested in 8 trials. The Shapiro-Wilk test (W = 0.982, p = 0.031) revealed that the overall data for ten inbred lines deviated significantly from a normal distribution. Thus I applied a Wilcoxon signed-rank test, which reveals that the overall Portugal **unconditioned inbred population exhibit a spontaneous preference for orange odor** compared to the chance level (V = 7862, p = 0.001) (Table 3.6).

Then I investigated the spontaneous odor preference of unconditioned flies for each inbred line comparing the proportion of orange choices for flies exposed to A/O and O/A (Figure 3.8). The Shapiro-Wilk test showed that the data for R7 were significantly not normally distributed when exposed to A/O (W = 0.755, p = 0.009) (Table 3.7). To test whether the spontaneous preference for orange odor was significantly different from the chance level when flies were exposed to A/O or O/A, I applied a Wilcoxon rank-sum test with Bonferroni-Holmes correction. These analyses reveal that R10 showed a significant preference for orange odor when exposed to O/A (V = 0.02, p = 0.024) but for flies exposed to A/O no inbred line showed a significant preference for orange odor compared to the chance level (Table 3.8). This could be due to a high variability because of a small starting sample size (40 flies per trail). Hence I would recommend to increase the sample size.
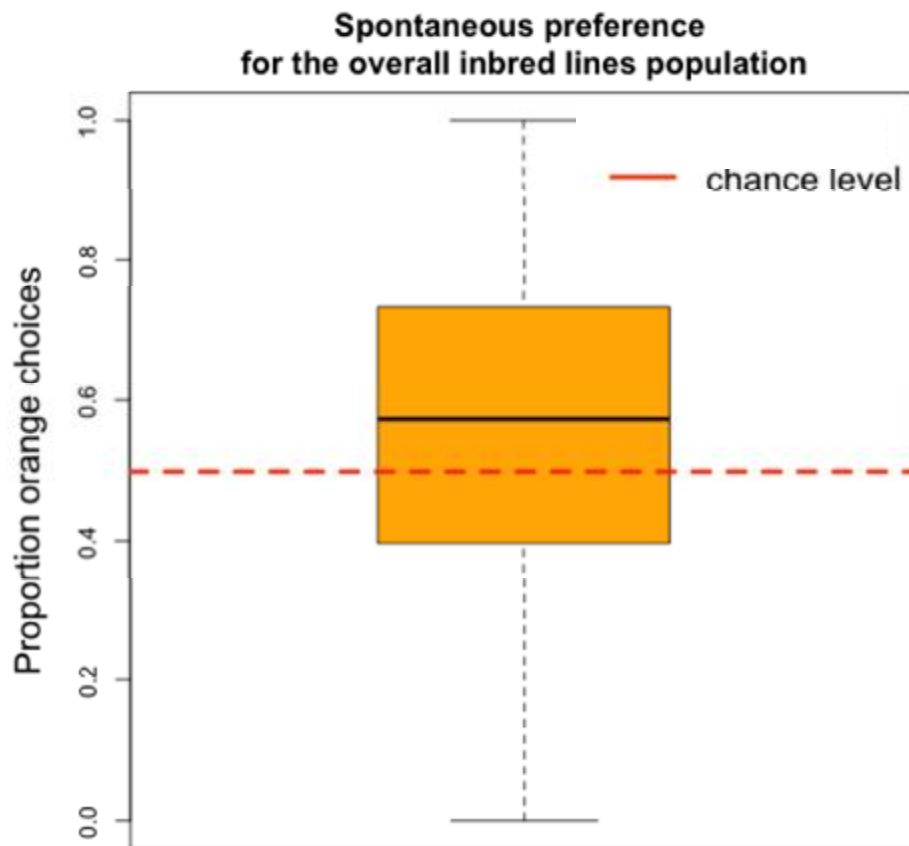
Figure 3.7. Spontaneous orange odor preference of the overall Portuguese *D. melanogaster* population.
This boxplot represents the proportion of orange choices of the entire unconditioned inbred population (Mean = 0.565, SE = 0.019). The chance level represents the expected value for the absence of any olfactory preference.

Table 3.6. Data analysis of the spontaneous preference for the entire population.

Orange odor choices for the entire population without dividing by the order of odor presentation (A/O and O/A)		
Statistical tests	**Shapiro-Wilk test**	**Wilcoxon signed-rank test**
Overall population	W = 0.982 p = 0.031	V = 7862 p = 0.001
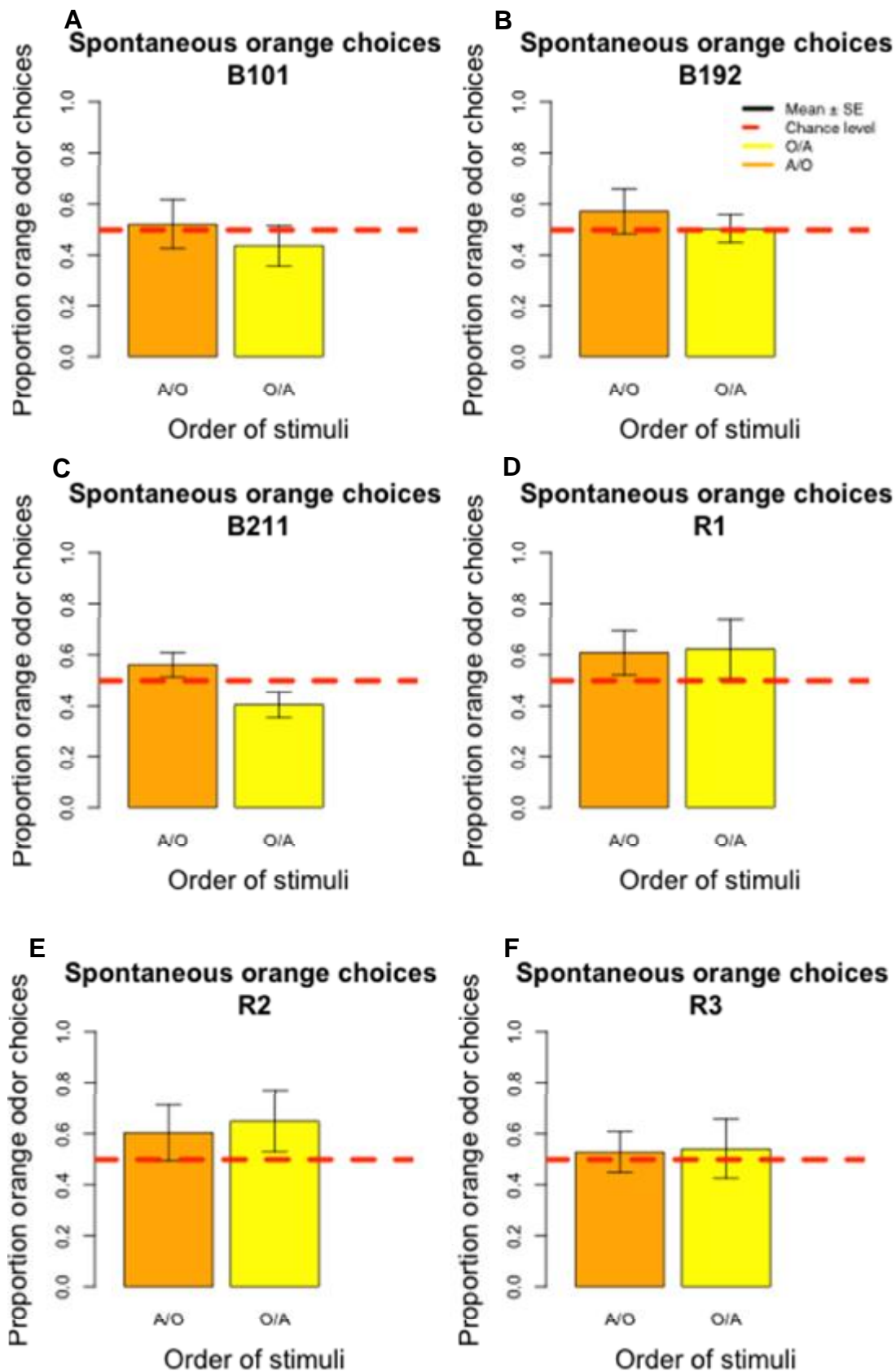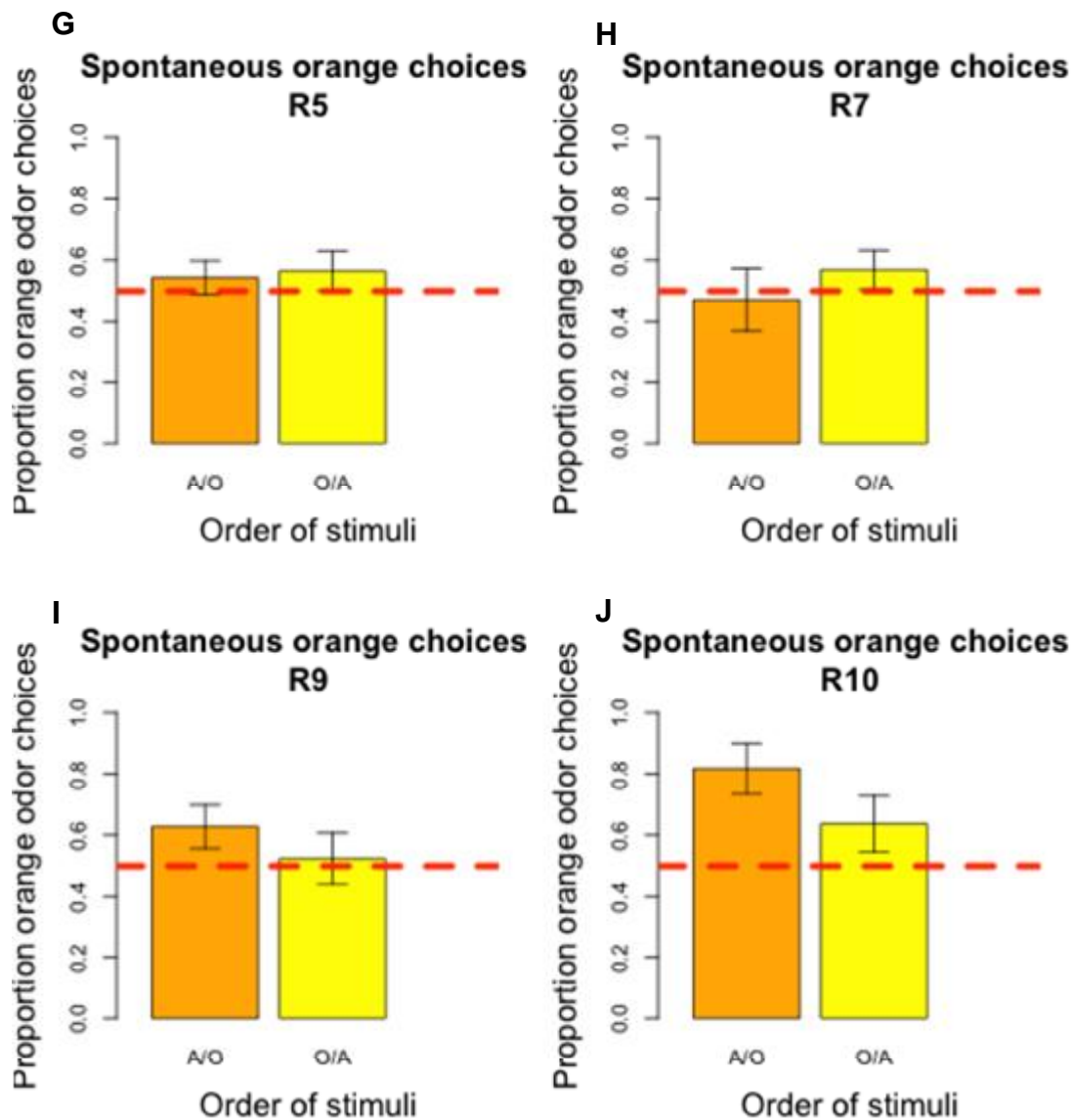
Figure 3.8. Proportion of orange odor choosers of unconditioned Portuguese *D. melanogaster* inbred lines.

A The orange bar (Mean = 0.52, SE = 0.10) shows the proportion of orange odor choices for B101 exposed to the A/O order and the yellow bar (Mean = 0.44, SE = 0.08) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

B The orange bar (Mean = 0.57, SE = 0.09) shows the proportion of orange odor choices for B192 exposed to the A/O order and the yellow bar (Mean = 0.50, SE = 0.06) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

C The orange bar (Mean = 0.56, SE = 0.05) shows the proportion of orange odor choices for B211 exposed to the A/O order and the yellow bar (Mean = 0.40, SE = 0.05) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

D The orange bar (Mean = 0.61, SE = 0.09) shows the proportion of orange odor choices for R1 exposed to the A/O order and the yellow bar (Mean = 0.62, SE = 0.16) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

E The orange bar (Mean = 0.60, SE = 0.11) shows the proportion of orange odor choices for R2 exposed to the A/O order and the yellow bar (Mean = 0.65, SE = 0.012) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

F The orange bar (Mean = 0.53, SE = 0.08) shows the proportion of orange odor choices for R3 exposed to the A/O order and the yellow bar (Mean = 0.54, SE = 0.12) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

G The orange bar (Mean = 0.54, SE = 0.05) shows the proportion of orange odor choices for R5 exposed to the A/O order and the yellow bar (Mean = 0.56, SE = 0.06) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

H The orange bar (Mean = 0.47, SE = 0.10) shows the proportion of orange odor choices for R7 exposed to the A/O order and the yellow bar (Mean = 0.57, SE = 0.06) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

I The orange bar (Mean = 0.63, SE = 0.07) shows the proportion of orange odor choices for R9 exposed to the A/O order and the yellow bar (Mean = 0.52, SE = 0.08) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

J The orange bar (Mean = 0.82, SE = 0.08) shows the proportion of orange odor choices for R10 exposed to the A/O order and the yellow bar (Mean = 0.64, SE = 0.09) represents the proportion of orange odor choices for unconditioned flies exposed to the O/A order.

Table 3.7. Data analysis for the spontaneous preference of orange odor for A/O exposure.

Orange odor choices for A/O exposure				
Inbred lines	**Shapiro-Wilk test**	**One Sample *t* test of A/O compared to 0.5**	**Wilcoxon rank-sum test compared to 0.5**	**Wilcoxon after Bonferroni-Holmes correction**
B101	W = 0.942 p = 0.633	t = -0.802 df = 7 p = 0.450	V = 12 p = 0.461	p = 0.461
B192	W = 0.914 p = 0.380	t = 0.081 df = 7 p = 0.94	V = 19 p = 0.945	p = 0.945
B211	W = 0.915 p = 0.388	t = -1.903 df = 7 p = 0.099	V = 5 p = 0.151	p = 0.151
R1	W = 0.901 p = 0.296	t = 1.060 df = 7 p = 0.325	V = 20 p = 0.352	p = 0.352
R2	W = 0.900 p = 0.291	t = 1.251 df = 7 p = 0.251	V = 20 p = 0.352	p = 0.352
R3	W = 0.967 p = 0.876	t = 0.348 df = 7 p = 0.738	V = 21.5 p = 0.674	p = 0.674
R5	W = 0.989 p = 0.993	t = 1.015 df = 7 p = 0.344	V = 19 p = 0.447	p = 0.447
R7	W = 0.755 p = 0.009	t = 1.097 df = 7 p = 0.309	V = 21 p = 0.742	p = 0.742
R9	W = 0.938 p = 0.590	t = 0.27 df = 7 p = 0.795	V = 20.5 p = 0.779	p = 0.779
R10	W = 0.912 p = 0.365	t = 1.496 df = 7 p = 0.178	V = 27 p- = 0.233	p = 0.233

Table 3.8. Data analysis for the spontaneous preference of orange odor for the O/A exposure.

		Orange odor choices for O/A exposure			
Inbred lines	**Shapiro-Wilk test**	**One Sample *t* test if A/O compared to 0.5**	**Wilcoxon rank-sum test compared to 0.5**	**Mean**	**Wilcoxon after Bonferroni-Holmes correction**
B101	W = 0.938 p = 0.592	t = 0.22 df = 7 p = 0.832	V = 22 p = 0.624	0.521	p = 0.624
B192	W = 0.862 p = 0.126	t = 0.830 df = 7 p = 0.434	V = 22 p = 0.641	0.572	p = 0.641
B211	W = 0.876 p = 0.171	t = 1.297 df = 7 p = 0.236	V = 23 p = 0.151	0.562	p = 0.151
R1	W = 0.932 p = 0.535	t = 1.259 df = 7 p = 0.248	V = 19 p = 0.446	0.609	p = 0.446
R2	W = 0.941 p = 0.618	t = 0.953 df = 7 p = 0.373	V = 19 p = 0.446	0.604	p = 0.446
R3	W = 0.887 p = 0.220	t = 0.347 df = 7 p = 0.739	V = 22 p = 0.624	0.528	p = 0.624
R5	W = 0.935 p = 0.566	t = 0.802 df = 7 p = 0.449	V = 21.5 p = 0.674	0.544	p = 0.674
R7	W = 0.988 p = 0.991	t = -0.281 df = 7 p = 0.787	V = 16 p = 0.844	0.471	p = 0.844
R9	W = 0.889 p = 0.231	t = 1.802 df = 7 p = 0.115	V = 29.5 p = 0.123	0.627	p = 0.123
R10	W = 0.819 p = 0.046	t = 3.808 df = 7 p = 0.007	V = 34.5 p = 0.024	0.816	p = 0.024

4 Discussion

Associative learning has been shown in many species including *Drosophila melanogaster* (Wen et al., 1997; Dukas and Bernays, 2000; Tryon, 1940). Due to the similarity of the function and anatomy of the olfactory nervous system to vertebrates, olfactory learning in *D. melanogaster* is a good model for learning (reviewed in Davis 2004). Learning and memory in *D. melanogaster* can have implications for human-related studies such as research on neurodegenerative diseases like Alzheimer disease and Parkinson's disease (Lenz et al., 2013). For this reason reliable and convenient methods to investigate behavioral traits in *D. melanogaster* may have socioeconomic relevance.

Learning and memory have a genetic component, as several studies have already shown (reviewed in McGuire et al., 2005). Nevertheless it is still not clear which allelic variants related to learning and memory are present in natural fly populations and what is the genetic architecture of these traits by studying the natural variation for learning capabilities. Some studies have already confirmed that natural variation of learning exists (Mery and Kawecki, 2002; Mery and Kawecki, 2003), showing that a natural population harbors enough standing variation to select for enhanced olfactory learning. Mery and Kawecki (2002) used the oviposition paradigm to select for enhanced olfactory learning. The main advantages of this method are that large groups of flies can be conditioned and tested (150 flies per trial) with a significant improved olfactory learning performance from generation 15 to 27. Although this method was effective in producing an increase of learning abilities in some generations of selection, nevertheless it has some disadvantages. The main disadvantages of the method are that it is very labor intensive because eggs have to be washed every generation before the propagation on standard medium. Furthermore the oviposition method can impose selection only for enhanced learning in females, not in males because eggs were selected only based on female performance. Another disadvantage is that scientists selected also for fast egg laying, because flies laid eggs for the subsequent generation during six hours of the testing phases. If one imposes selection at the same time for enhanced learning and for other traits such as fast egg laying and egg resistance to washing, the genomic response to learning will be confounded and potentially biased. Thus, it is necessary

to develop a method, which overcomes these drawbacks if genes are sequenced. Moreover, a cheaper and faster method can enable researchers to condition and test large groups of flies with selection only on olfactory learning performance to run extended experimental evolution studies matched with resequencing at different timepoints (Evolve&Resequence: E&R) or GWAS.

It is very important to have a large population size and many replicates for GWAS and for E&R further many generations to identify selected loci used for learning (Kofler and Schlötterer, 2013; Bastide et al., 2013). For these reasons I have developed and tested a new method to investigate learning in large groups of *Drosophila*. In my thesis I show that I have established the new procedure to condition and phenotype large populations of flies in olfactory and other sensory modalities, for instance vision. Moreover, the method is also suitable to assay spontaneous olfactory preferences. Assaying the spontaneous preference of the experimental stimuli used for learning experiments is necessary because learning depends on perception and consequently the capability to discriminate between the experimental stimuli. Thus, it is important to analyze whether perception changes during selection for learning because this would influence the learning performance. Moreover, the method can be used also to investigate perceptual preferences independently of learning by easily adjusting the procedure and going directly to the test phase.

During my master thesis project I worked in a team interested in establishing a convenient method to investigate learning in fruit flies. I first developed a T-maze, which consists of parts found in a common biology lab. Then I worked at the details of a conditioning procedure consisting of two different exposures followed by a starvation phase and a test phase. During the exposure phases flies should learn to associate an odor with an aversive flavor and a second odor with a palatable flavor.

Using the established procedure I performed several experiments to investigate whether this method is suitable to investigate olfactory learning and olfactory preferences. In particular, I investigated whether, using this method, flies can learn to avoid the odor previously associated with aversive flavor and significantly choose the odor previously associated with palatable taste during the test phase. I also

investigated whether this method is suitable to analyze spontaneous preferences for different olfactory stimuli (orange and apple juice odor).

In my thesis I describe the developed method (section 2.2.2) and the experimental findings for olfactory learning (section 3.1.1 and 3.2.1) and spontaneous odor preference (section 3.1.2 and 3.2.2). Then, I compare the T-maze method with the oviposition paradigm (Mery and Kawecki, 2002) and other methods previously used in the field (section 1.2.2 and 4). Finally, I describe the potential of this method for future research (section 4).

In a first series of experiments I tested a large isofemale population, which consists of inseminated female *D. melanogaster* derived from a natural fly population caught in South Africa whereas each female is separated in a vial and its offspring is allowed to mate. This isofemale population is likely to harbor many natural learning variants.

Testing a large isofemale population was relevant to investigate if large groups of flies with diverse genetic backgrounds were able to perform olfactory learning after conditioning with the developed conditioning procedure. I showed that using my methods a large population derived from South Africa was able to learn the association between an odor (orange juice odor) with appetitive flavor and another odor (apple juice odor) with aversive flavor. Flies were able to recall the formed memory during the test because they significantly increased the choice for the odor previously not associated with aversive taste. Since this effect was stronger when flies were conditioned to choose orange compared to flies conditioned to choose apple, it would be more effective and useful to apply conditioning to choose orange odor and choose orange odor for selection experiments of improved learning.

I also investigated whether flies improved their learning performance after subsequent conditioning. For this experiment I repeated the same procedure for two consecutive days to investigate if the learning performance improved after an additional conditioning. Interestingly flies showed no significant improvement of the learning performance after a subsequent conditioning compared to flies conditioned during one day. In spite of this I observed a trend towards an increasing learning performance from day 1 to day 2. This observation suggests that there was probably not enough statistical power to detect a significant learning result due to a decrease of starting sample from day 1 to day 2. Thus to improve this procedure it would be

necessary to start with a larger sample size to increase the statistical power. The T-maze allows testing more than 250 flies per trial, thus this adjustment is easily feasible. I calculated that the starting sample in day 1 should increase from 250 flies per trail to about 425 flies per trial to obtain a sufficient amount of choosers also in day 2.

To date, researchers in molecular biology (reviewed in McGuire et al., 2005; Davis, 2004; Davis, 2005) and evolutionary genetics (Swarup et al., 2013; Wang, et al., 2010; Brown et al., 2013; Steck et al., 2012; Buck and Axel, 1991) have used olfactory behavior as a model to investigate perception. Beside the general interest in spontaneous olfactory behavior, it is important to investigate the spontaneous preference for odors used during the learning procedure because learning depends on perception. Recently Dweck et al. (2013) studied the evolutionary bases of the spontaneous preferences for ovipositing substrates in *D. melanogaster.* They found that flies preferred to oviposit on citrus fruits compared to other fruits. Furthermore they found that the citrus preference is an ancestral trait, due to adaptation to fruits occurring in the original habitat Africa. Parasitic wasps, a natural *D. melanogaster* parasite, avoids the odor of citrus thus Dweck et al. 2013 concluded that the observed preference for citrus odor is likely to be a strategy of *D. melanogaster* to protect their offspring from parasitism. The egg-laying preference of *D. melanogaster* was tested using a multiple-choice oviposition assay. In this assay 30 flies per trial were offered the choice to oviposit on six different fruits during 24 hours. The main advantages of this multiple-choice oviposition assay are that one can investigate the spontaneous preference of six different fruits at the same time and that the flies have no prior experience with these odors. The main disadvantage of this assay is that one can only study the spontaneous preference of females not males because of the ovipositing performance. Another disadvantage is that the method is very time-consuming because eggs laid on the fruits have to be counted. Another disadvantage is that one cannot control for the number of eggs laid by different females concluding to a biased olfactory preference recording. Other methods have been used to test odor preferences in flies (Mery and Kawecki, 2002; Dweck et al., 2013; Stensmyr et al., 2012) but they are limited to test a small number of flies (Mery and Kawecki, 2002) or are very time consuming (Dweck et al., 2013; Stensmyr et al., 2012). Thus it would be important to find a method, which overcomes these drawbacks and that is

fast and simple enough to test large groups of flies in a limited amount of time. A method similar to the one I used in my learning assays can be used to this aim. In my spontaneous odor preference assay flies were exposed to two different odors (orange and apple juice odor) each of them provided in one side chamber of the T-maze. 250 flies had the chance to associate each odor with palatable flavor during two exposure phases. After starvation flies had the choice to choose one odor during the test phase. I used the same multiple exposure procedure as applied for the conditioning because I wanted to compare the spontaneous preference for the exposure order with the learning results. I repeated the procedure for a consecutive day to investigate if the preference changes after an additional exposure day. Analyzing the spontaneous preference of the South African population revealed that this population showed a preference for orange odor compared to apple odor. This result shows that the procedure is sensitive enough to detect the spontaneous preference difference between orange and apple odor at the population level. The preference for orange odor was consistent with the results of the citrus odor preference for ovipositing substrates of *D. melanogaster* due to protection of parasitic wasps (Dweck et al., 2013). A similar orange odor preference had been found also in the fly population used for experimental evolution in olfactory learning (Mery and Kawecki, 2002). The method can be easily adjusted to study the spontaneous preference independently of the learning procedure, by applying directly the test phase and hence reducing the effort in phenotyping.

In a second series of experiments I investigated if genetic differences of learning exist, testing eleven isofemale lines of *D. melanogaster* derived from Portugal and inbred for some generations (17 to 29 generations). To this aim I used isofemale lines inbred for several generations to ensure that those inbred lines consist of the same genetic background. Variation of the olfactory learning performance among all inbred lines maintained with the same regime would indicate that there are genetic variants different between lines that can affect learning. I applied the newly developed method to condition small groups of flies (40 flies per trial). One line was not responding to the procedure (did not make any choice), thus I excluded it from data analyses. The overall population exhibits a significant learning performance showing that the population learned the association of an odor with appetitive or aversive taste. Then I analyzed the learning performance of each inbred line. Five out

of ten remaining lines significantly learned the association between a taste and aversive or appetitive taste and they were able to recall the acquired memory during the test (R1, R2, R3, R7, R9). These significant learning results were obtained during only eight trials with only 40 flies per trial. This suggests that the conditioning procedure is very effective and also suitable to condition smaller groups of flies.

The inbred lines showed no significant preference for orange or apple odor on the individual level, but interestingly the overall population exhibited a preference for orange odor. This discrepancy could be due to an increase of statistical power at the population level. Again, the orange preference was consistent with the preference of the South African population and the citrus odor preference (Dweck et al., 2013).

Concluding, the new developed procedure is a cheap and very effective method to condition large groups of flies and single lines, and it is also suitable to test the spontaneous preferences. Compared to the oviposition paradigm (Mery and Kawecki, 2002) the method I have established is superior because it is less labor intensive and the learning performance is not biased by other traits such as fast egg laying or egg resistance to washing. Nevertheless there is also a possibility to improve my method. I would recommend to increase the number of flies in each trial (> 250 flies for individual lines, 425 for investigating a population: see page 39) to increase the statistical power to detect preferences. This improvement is easily feasible, given that the vials can be used with larger sample size.

As shown in this master thesis the established olfactory learning procedure provides a possibility to observe a significant effect of learning in the absence of selection. This adds to growing evidence about learning capabilities in flies (Mery and Kawecki, 2002; Mery and Kawecki, 2003; Durisko and Dukas, 2013; Plaçais and Preat, 2013; Dudai et al., 1976; Heisenberg et al., 1985; Tully and Quinn, 1985; Tanaka et al., 2007; Guan et al., 2011; Mery et al., 2007; Zrelec et al., 2013; Dill and Heisenberg, 1995; Hollis et al., 2014; Babin et al., 2014). Furthermore it is possible to select for improved learning without selection of confounding factors compared to the oviposition paradigm (Mery and Kawecki, 2002).

The new method can be applied also in other sensory modalities like vision, and other domains such as phototaxis or spatial navigation, providing a possibility to broaden the investigation of learning capabilities and spontaneous preferences.

In fact it has already been shown that flies are able to remember visual patterns (Dill and Heisenberg, 1995) and they can associate green or blue color with heat (Wolf and Heisenberg, 1997). Applying the new method one could investigate if flies are able to perform visual learning by associating a color with palatable flavor and another color with aversive flavor. With this method it should be also possible to test if flies have a spontaneous preference for different colors. To condition flies for visual learning, I would add two different colors to the side chambers of the T-maze.

The method can be applied also beyond behavioral research. In fact it can be useful for E&R and GWA studies because one can condition large groups of flies simultaneously. E&R is a combination of experimental evolution studies with sequencing of multiple individuals at different time points during selection and provides a possibility to investigate the development of complex traits such as learning capabilities during evolution (Schlötterer et al., submitted). E&R provides the possibility to determine selected loci and differences in allele frequencies between different selection experiments when investigating a large population size with many replicates for many generations. Nevertheless E&R and GWAS using a large population size and many replicates are more expensive but they produce reliable and fine-scale genomic data compared to cheaper experiments with few replicates and a small population size (Kofler and Schlötterer, 2013). E&R and GWAS combined with the new procedure could shed light on the natural variability of learning and preferences in different domains and sensory modalities.

5 Acknowledgment

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6 Zusammenfassung

Das Hauptziel dieser Masterarbeit war es eine Methode zu entwickeln, die es ermöglicht mit geringem Aufwand eine große Anzahl an Fruchtfliegen (*Drosophila melanogaster*) zu konditionieren. Dabei sollen Fliegen die Assoziation zwischen einem Geruch (Orangensaftgeruch) mit wohlschmeckenden Futter und einem anderen Geruch (Apfelsaftgeruch) mit bitterem Futter lernen. Tatsächlich lernten die Fliegen den Geruch, der mit bitterem Geschmack assoziiert wurde, während des Testes zu vermeiden. Sie entschieden sich stattdessen signifikant für den Geruch der zuvor mit gutem Geschmack assoziiert wurde. Dieses Ergebnis zeigt, dass die entwickelte Methode geeignet ist, um große Gruppen an Fliegen (250 Fliegen pro Versuch) mit vergleichsweise geringem Aufwand zu konditionieren (Mery und Kawecki, 2002). Eine weitere Anwendungsmöglichkeit der etablierten Methode ist es, angeborene Präferenzen für Gerüche wie Orangen- oder Apfelgeruch von *D. melanogaster* zu erforschen. Diese Arbeit zeigt, dass die unkonditionierte Fliegenpopulation Orangensaftgeruch im Vergleich zu Apfelsaftgeruch signifikant bevorzugte. Dieses Ergebnis ist konsistent mit der Beobachtung einer Publikation, die zeigte, dass *D. melanogaster* eine angeborene Präferenz für Orange als Substrat für die Eiablage besitzt (Dweck et al., 2013).

Ein weiteres Ziel dieser Arbeit war, das Vorkommen genetischer Variabilität von olfaktorischem Lernen zu erforschen. Dazu wurden zehn Inzuchtlinien (40 Fliegen pro Versuch) konditioniert. Sechs dieser Inzuchtlinien waren im Stande die Assoziation von Orangensaftgeruch mit gutem Geschmack und Apfelsaftgeruch mit bitterem Geschmack zu lernen. Dieses Ergebnis zeigt, dass sechs Inzuchtlinien im Stande waren zu lernen, und dass genetische Variabilität in olfaktorischem Lernen vorhanden ist. Interessanterweise zeigte keine der Inzuchtlinien eine signifikante Präferenz für Orangen- oder Apfelsaftgeruch. Das liegt vermutlich an der hohen Variabilität, die auf Grund der geringen getesteten Fliegenanzahl zu Stande kommt.

Diese neue Methode erlaubt es, die natürlich vorkommende Variation an Genen, die für das Lernen benötigt werden, mit Hilfe von E&R als auch GWAS Studien zu erforschen. Weiters erlaubt die Methode auch die Untersuchung von anderen Sinnesmodalitäten, wie visuelles Lernen oder angeborene Farbpräferenzen.

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26th of June 2014

Julia Reisenberger

Protocol

This protocol provides a detailed breakdown of all necessary steps and needed materials to repeat the olfactory learning experiments described in my master thesis.

Materials

Please make sure to you use the appropriate safety data sheets and safety equipment for proper handling of hazardous reagents and equipment used in this protocol.

Reagents

orange and apple juice from concentrate (from home brand Spar)

agar-agar

organic malt syrup (brand TerraSana)

nipagine

ethanol

propionic acid

quinine hydrochloride

Equipment

cooking spoon

plastic measuring cylinder

glass measuring cylinder

scale

microwave oven

pipette

standard *Drosophila* fly food vial

net

white trays to store vials and perform experiments (40 x 30 x 8.5 cm)

fridge

incubator

neon lamps (Ultra Slim T4/20W/G5 with 50 Hz)

anesthesia flypad (brand Flystuff)

Egg counter

brush for collecting flies

carton dividers

T-maze (31 x 17.5 x 1.5 cm)

Whatman paper

Method

Preparing the experimental food

Cooking fly food with orange and apple flavor for the olfactory experiments should be done by following this recipe:

Appetitive medium use gloves!		Aversive medium use gloves!	
0.5 L	orange or apple juice from concentrate from home brand Spar *use a plastic measuring cylinder.* *You should always use the*	0.5 L	orange or apple juice from concentrate from home brand Spar *use a plastic measuring cylinder.* *You should always use the*

	total juice pack, when you store half of the pack in the fridge, it might have a different taste.		*total juice pack, when you store half of the pack in the fridge, it might have a different taste.*
+ 7 g	agar-agar	+ 7 g	agar-agar
+ 60 ml	organic malt syrup from TerraSana *use a glass measuring cylinder* *Syrup shouldn't stick to the sidewalls of the plastic measuring cup, when you add it.*	+ 60 ml	organic malt syrup from TerraSana *use a glass measuring cylinder* *Syrup shouldn't stick to the sidewalls of the plastic measuring cup, when you add it.*
	• bring to boil *set the microwave to full power for 5:30 to 6:00 min, be careful it might overboil.* • stir with a cooking spoon or similar • cool down for at least 5 minutes before you add solubilized nipagine and propionic acid.		• bring to boil *set the microwave to full power for 5:30 to 6:00 min, be careful it might overboil* • stir with a cooking spoon or similar • cool down for at least 5 minutes before you add solubilized nipagine, propionic acid and quinine.
+ 1 g + 2.5 ml	nipagine solubilize in ethanol *use a scale to measure 1 g and a pipette to measure 2.5 ml* *shake it carefully, all nipagine should be solubilized in ethanol*	+ 1 g + 2.5 ml	nipagine solubilize in ethanol *use a scale to measure 1 g and a pipette to measure 2.5 ml* *shake it carefully, all nipagine should be solubilized in ethanol*
+ 2 ml	propionic acid *When breathing in, it can*	+ 2 ml	propionic acid *When breathing in, it can*

	irritate your respiratory tract.		*irritate your respiratory tract.*
		+ 4 g	quinine hydrochloride *use a scale*
	stir and pour 4 ml into standard *Drosophila* vials. *1.5 cm of food should be in each vial, the amount should be similar in each vial.*		stir and pour 4 ml into standard *Drosophila* vials. *1.5 cm of food should be in each vial, the amount should be similar in each vial.*
	Covering trays with a mosquito net prevents wild flies of contaminating fresh food. Vials should dry about 5 hours before plugging food.		Covering trays with a mosquito net prevents wild flies of contaminating fresh food. Vials should dry about 5 hours before plugging food.
	Label trays with date and **+** for appetitive food. Store trays in the fridge at 4 °C for maximum 2 weeks.		Label trays with date and **-** for aversive food. Store trays in the fridge at 4 °C for maximum 2 weeks.

I Olfactory learning population experiment

In this experiment I am interested to test whether a newly developed olfactory learning paradigm can be used to test large groups of flies from a natural population of *D. melanogaster*. This population consists of 670 isofemale lines collected in South Africa 2012.

In this procedure, after starvation flies are conditioned to associate either apple or orange odor with aversive (Exposure 1 phase) and subsequently appetitive taste (Exposure 2 phase). After conditioning flies are tested for learning (Test phase).

1. Flies collection

1) *17 hours* before starting the experiment, collect 250 flies for each experiment. An easy and fast way to collect them is to align the flies on the anesthesia flypad and use a counter. Use flies that are at least 24 hours old, as evident from their pigmentation.

In my experiments I used 250 flies from a South African population of D. melanogaster but it is possible to increase this number. With 250 flies I had on average of 96 flies for the test session during the first day and 53 flies for the test session during the second day. With this number one can assume that the sex ratio is about 50:50. Collect only a few flies (4) from each line (not more than 10 from a single line).

2) Transfer 250 flies for each experiment into a room-temperature fresh standard fly food vial.

On a typical day I was able to run experiments using 10 to 12 apparatuses.

3) Keep flies in the incubator before starvation.

Following settings are used for the incubator: light from 8:00 to 22:00 and darkness from 22:00 to 8:00 with a constant temperature of 22 °C (same temperature used for the experiments).

2. Starvation 1

4) 15 hours before the experiment, flip flies in a vial supplied with a piece of moistened Whatman paper for starvation.

Moistened Whatman paper prevents desiccation (make sure it is not too wet, otherwise flies are stuck on the water drops).

5) Put the vials with starving flies in the incubator for 15 hours overnight.

3. Prepare apparatuses before starting the experiments

Always use the same number of apparatuses with apple and orange as aversive stimulus. This will help to control for batch effects.

Number the apparatuses (1, 2, 3, 4…) and work on them always maintaining the same order: this will help to be more systematic and accurate in collecting and recording data.

Each apparatus (see Figure 1) is placed in a white opaque box (40 x 30 x 8.5 cm), in this way flies are not influenced by flies tested in other apparatuses. *Make sure to have equal lightening on both sides of the T (check the shadows). Flies have a strong positive phototaxis, and would prefer the vial with more light.* Neon lamps (Ultra Slim T4/20W/G5 with 50 Hz) are placed at 41 cm distance on top/center of each apparatus. Cardboard dividers separate neon lights from each other, to guarantee homogeneous lightening in each apparatus.

6) Add two aversive food vials to each apparatus: two vials of bitter orange food to a T-maze and two vials of bitter apple food to the other T-maze placed under one neon light.

During Exposure 1 phase, in half apparatuses apple juice food is supplemented with quinine and in the other apparatuses orange juice food is supplemented with quinine.
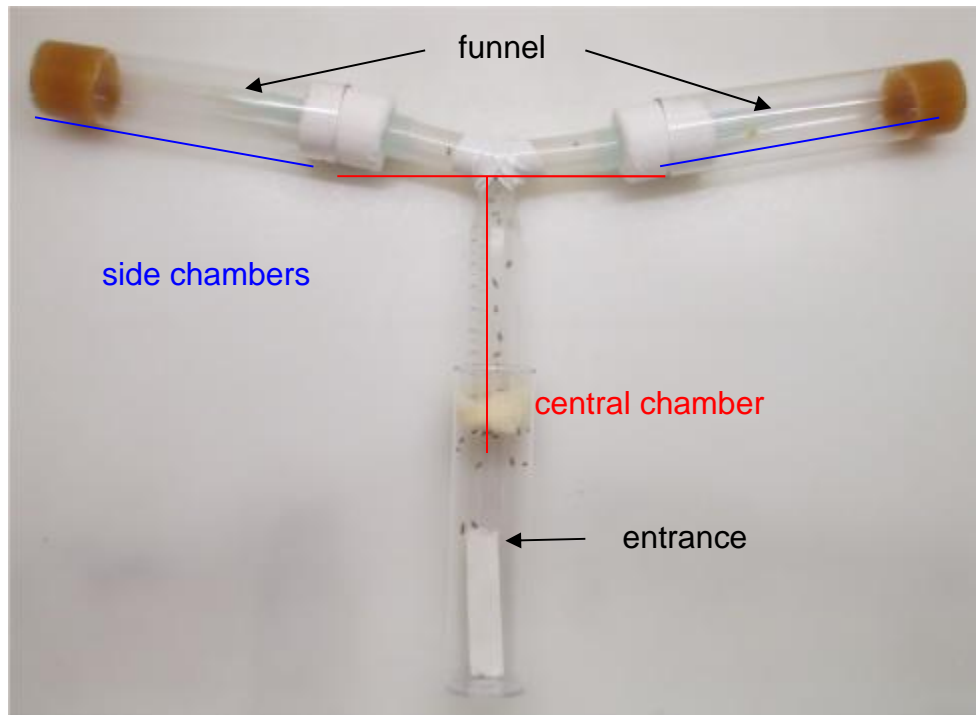
Figure 1. Olfactory phenotyping T-maze.
The T-maze (31 x 17.5 x 1.5 cm) used for olfactory phenotyping consists of a transparent central T-chamber (12 x 8 x 1.5 cm). At the entrance of the T-maze a standard *Drosophila* vial (9.5 x 2.5 cm) with starved flies can be connected using a sponge. On both ends of the T-maze a standard *Drosophila* vial filled with 4 ml of orange or apple juice medium is located representing the side chambers. At the beginning of each period supply fresh media to the apparatus. Connect the central chamber and the vials containing fly food with a narrow funnel made with a 1000 µl pipette tip cut at the edge. In this way flies will be able to enter the fly food vials, but once inside and close to the food it will be extremely unlikely for them to escape.

4. Count dead flies

7) For each apparatus count how many flies die during starvation and record these data.

5. Start experiment

After having added the side chambers with food wait 10 minutes because the odor should have time to distribute through the T-maze. Then connect a vial containing starved flies to the apparatus using a sponge. Proceed from apparatus 1 to apparatus 2 and so on, always with the same serial order.

The starting chamber is connected to the central chamber using a sponge and help yourself with the tip of a brush or similar object. *After connecting all vials to the apparatuses, check if everything is connected properly and flies can't escape.*

5.a. <u>EXPOSURE 1</u> phase, aversive food exposure (2 hours)

In these 2 hours most of the flies should enter the vials containing food and experience either orange or apple bitter flavor. They are trapped in the vials containing food through the funnel.

8) Flies trapped in the side chambers are collected and transferred in another empty vial. Do this for all the apparatuses in serial order (apparatus 1, 2, 3…). These flies will be subsequently moved to Exposure 2 phase.

9) Discard the aversive food vials.

Flies can smell eggs and pheromones of previous flies, for this reason used vials have to be discarded.

10) All flies remaining in the T-chamber are discarded.

They had no chance to associate the bitter taste with a smell.

5.b. Prepare apparatuses for Exposure 2 phase

11) Connect 2 vials of orange appetitive food to half of the apparatuses and 2 vials of apple appetitive food to the other half, 10 minutes before connecting vials with flies to the apparatuses. Apparatuses that before contained apple now will contain orange and vice versa.

5.c EXPOSURE 2 phase, appetitive food exposure (2 hours)

In Exposure 2 phase most of the flies should enter the vials containing food and experience either orange or apple odor with appetitive flavor.

12) Connect vials with flies to the apparatuses serially.

Make sure that flies previously associated orange with bitter taste, have now the chance to associate apple with appetitive taste and vice versa.

13) Flies trapped in the vials with appetitive flavor are collected and transferred in another empty vial. Do this for all the apparatuses serially. These flies, which have experienced orange with aversive taste during Exposure 1 phase and apple with appetitive taste during Exposure 2 phase and vice versa, will be used in the Test phase.

14) Discard the used vials.

15) All flies remaining in the T-chamber are discarded.

5.d Starvation 2 (4 hours)

16) Add a moistened Whatman paper to each vial containing flies that entered the vials with food in both phases, Exposure 1 and Exposure 2.

17) Starve the flies the next 4 hours.

After starvation flies are more motivated to look for food.

6. Prepare T-maze for test

18) Prepare the T-maze 10 minutes before testing. Add one orange appetitive food vial and one apple appetitive food vial on each apparatus. Half of the apparatuses will have orange on the right side and half on the left side.

6.a Test (1 hour)

19) Connect vials containing conditioned starved flies to the test apparatuses in the usual serial order (apparatus 1, 2, 3…).

20) Change food vials after 30 minutes and add new vials to the apparatuses.

This and the funnel reduces the chances that flies can make a second choice.

21) After additional 30 minutes finish the test and count flies (see below).

6.b Counting flies

22) Record how many flies are trapped in orange and apple food during the first and the second test.

The best way to record the number of trapped flies during the test is, to label the bottom of each food vial in advance with number of experiment, odor presentation order and the number of test. Use the anesthesia flypad to count how many flies are trapped in each vial.

7. Repeat conditioning assay for a second day

To test if flies improve their learning performance after a subsequent conditioning day, flies trapped in aversive and appetitive food during the test are collected then pooled together and starved for 15 hours. The conditioning procedure is repeated the next day.

8. Update the excel file

23) When you finish the experiments update all recorded data into an excel file. Below is an example with detailed descriptions for each column.

Date:	Date of experiment
Fly type:	Exact maintenance description of flies: species, population, temperature in incubator
Temp degree:	Temperature in the room in which the experiments are performed
Humidity percentage:	Humidity in the room in which the experiments are performed
Page side:	Page side in the experimental book, where data are recorded
Cage number:	Number of the cage (white box)
Run:	Run number (progressive incremental number)
Short Fly type:	Short form of the fly type (SA = South African population)
N start:	Number of start flies
N start after starvation:	Number of flies survived after starvation
Hours starvation:	Number of hours of starvation
Dead after starvation:	Number of dead flies after starvation
Prop dead after starvation:	Proportion of dead flies after starvation (total dead flies/total number of start flies)
1st exposure aversive:	Hours during Exposure 1 phase (aversive food)
2nd exposure appetitive:	Hours during Exposure 2 phase (appetitive food)
Plus stimulus:	Odor during Exposure 2 phase
Test 1 right choice:	Number of flies making the right choice during the first half an hour of the test. If apple is plus stimulus, apple choosers make the right choice.
Test 1 wrong choice:	Number of flies making the wrong choice during the first test. If apple is plus stimulus, orange choosers make the wrong choice.
Test 2 right choice:	Number of flies making the right choice during the second half an hour of the test.
Test 2 wrong choice:	Number of flies making the wrong choice during the second test.
Total good learners:	Sum of flies making the right choice
Prop good	Proportion of good learners compared to total choosers (total

learners:	number of good learners/total number of choosers)
Total poor learners:	Sum of flies making the wrong choice
Prop poor learners:	Proportion of poor learners compared to total choosers (total number of poor learners/total number of choosers)
Total orange choices:	Sum of all orange odor choosers during test 1 and test 2
Prop apple choosers:	Proportion of apple choosers compared to total choosers
Prop orange choosers:	Proportion of orange choosers compared to total choosers
Total choosers:	Total number of flies choosing during test 1 and test 2

9. Perform data analysis using R

II Spontaneous preference for population experiment

The goal of this experiment is to investigate whether flies have a spontaneous preference for one of the experimental stimuli (orange or apple odor).

The procedure of this assay is very similar to the previously explained olfactory learning population experiment. The only difference is that no aversive food is present in the Exposure 1 phase.

III Olfactory learning in *D. melanogaster* inbred lines

The goal of this experiment is to investigate the presence of genetic differences in olfactory learning between different flies of *D. melanogaster*. I used eleven inbred lines, derived from a natural population from Portugal. Groups of flies from each line have been conditioned to associate either apple or orange odor with aversive or appetitive taste and then I have tested their learning performance.

1. Collect flies

1) 17 hours before starting the experiment collect 2 x 20 flies from each inbred line separately. Make sure to have 20 females and 20 males of each line, older than 24 hours.

I was using only 40 flies, because there were not more flies available, but if more flies are available it would be better to use more flies. With 40 flies I had on average of 21 flies for the test session.

2) Transfer the 40 flies for each line into a room-temperature fresh standard fly food vial for at least 2 hours before starving.

Normally I was able to run 10 to 12 apparatuses per day, which equals 5 or 6 lines.

3) Keep flies in the incubator before starvation.

Following settings are used for the incubator: light from 8:00 to 22:00 and darkness from 22:00 to 8:00 with a constant temperature of 22 °C (same temperature used for the experiments).

All other steps are similar to the olfactory learning population experiment described before, except of the test in section 6.a. The procedure of the test is similar, but the length is different. The **test for inbred lines** should last **1.5 hours**, because fewer flies are conditioned compared to the population experiment.

To make sure to have as much choosers as possible, I give them more time to choose during the test.

IV Spontaneous preference in Portuguese inbred flies

Here I am interested if inbred lines have a spontaneous preference for one of the experimental stimuli. This assay is very similar to the procedure of the olfactory learning in Portuguese inbred flies, with the difference that there is no aversive food in Exposure 1 phase.