

Insect Olfactory Learning in the Context of Feeding and Oviposition

Dissertation

To Fulfill the Requirements for the Degree of
“Doctor of Philosophy” (PhD)

Submitted to the Council of the Faculty
of Biological Sciences
of Friedrich Schiller University Jena

by **Elisabeth Adam, MSc**

born on September 10th, 1984 in Judenburg, Austria

Reviewers

1. Prof. Dr. Dr. h. c. Dr. h. c. Bill S. Hansson, HonFRES, FAAS
Max Planck Institute for Chemical Ecology, Jena, Germany
2. Prof. Dr. Holger Schielzeth
Friedrich Schiller University, Jena, Germany
3. Prof. Thomas C. Baker, PhD
PennState College of Agricultural Sciences, University Park, Pennsylvania, U.S.A.

Date of public defense July 28th, 2022

"Anyone who stops learning is old, whether at twenty or eighty.

Anyone who keeps learning stays young."

Henry Ford

Dedicated to my parents...

Elisabeth Adam

the ultimate teacher, who taught me how to learn and memorize.

Eligius Adam

a role model growing up, who taught me that it's never too late to follow your calling.

Table of Contents

Introduction	7
Learning in Insects	7
Testing Insect Learning in the Laboratory.....	7
Insect Olfaction and Olfactory Learning.....	9
The Tobacco Hawkmoth	11
Methodology: Behavioral Experiments in the Wind Tunnel	13
Aim of This Thesis.....	14
The Context of Foraging	14
The Context of Oviposition.....	14
One Trial Learning	14
Manuscript I: Moths Sense but do not Learn Flower Odors With Their Proboscis During Flower Investigation.....	15
Manuscript II: Host Plant Constancy in Ovipositing <i>Manduca sexta</i>	28
Manuscript III: Fast Learners: One Trial Olfactory Learning in Insects	37
Discussion	46
Discussion of The Context of Foraging (Manuscript I)	46
Hawkmoth Ecology and Odor Learning.....	48
Instant Reward Increases Foraging Motivation	49
Is the Proboscis a Gustatory Organ for the Quality Assessment of the Flower?.....	50
Discussion of The Context of Oviposition (Manuscript II).....	51
The Ovipositor: Involved in Olfactory Host Leaf Learning?.....	51
One Trial and Prepared Learning in The Context of Oviposition	52
Motivation and Oviposition.....	53
Discussion of One Trial Learning (Manuscript I-III).....	53
How to Improve Studies on Olfactory Learning in Insects	54
Setting the Results of Manuscript I & II into Perspective	55
Conclusion	55
Summary	56
Zusammenfassung	57
References	58
Declaration of Independent Assignment.....	65
Acknowledgements.....	66
Appendix.....	69

Introduction

“Live as if you were to die tomorrow. Learn as if you were to live forever.”

Mahatma Gandhi

As humans we look at this quote with an anthropocentric view. We expect to live the next day, year or even decade and we have to be reminded that life can be unpredictable. Learning for us seems like a luxury. We can do it at our own free will, but if we do not choose to continuously learn throughout our life there will hardly be any repercussions. Other species, especially those lower on the food chain, do not have the same luxury. For them learning and adapting to the environment is key for survival.

Learning in Insects

Despite their small size and the even tinier size of their central nervous system (i.e. brain) even very small insects have evolved the ability to learn (Busto et al., 2010, Giurfa, 2013, Smid et al., 2007, van der Woude et al., 2018, van Lenteren and Bakker, 1975). Their short lifespan should not fool one into thinking that learning might be irrelevant. Learning often only requires seconds or minutes and therefore is still advantageous even if the life span is comparably short (Dukas, 2008). But what does “learning” actually mean? Learning is defined as a “repeatable change of behavior due to experience”. It often includes a “learning curve” – gradually changing behavior with continued experience – and is plastic and shapeable when it comes to new experiences or forgetting (Papaj and Prokopy, 1989). Insects learn in all areas of life from foraging, to predator avoidance to social interactions and reproduction (Dukas, 2008). In the past, it was believed that all these behaviors are instinct-driven or innate (Fabre, 1918). Since then, research has discovered that insects do not only learn simple associations, but are also capable of learning concepts, learning from others or innovating, which are considered more complex learning abilities (Lihoreau et al., 2019). One should, however, be careful to interpret such abilities as “highly cognitive”, since many behaviors can be explained more parsimoniously by elemental (i.e. simple) associative learning (Giurfa, 2013).

Testing Insect Learning in the Laboratory

When investigating insect learning in the lab, the training usually includes the association between an unconditioned stimulus (US), such as a sugar reward or an electric shock, and a conditioned stimulus (CS). The CS that is selected should be innately neutral and can for

example be a color (visual), odor (olfactory), or sound (auditory) (Hammer and Menzel, 1995, Kahsai and Zars, 2011, Menda et al., 2011). Training the insect to associate the CS with the US is called “conditioning”. One differentiates between appetitive (rewarding) and aversive (punishing) conditioning (**Figure 1**). If the CS is combined with an appetitive US, an insect that is able to learn will henceforth be attracted to the CS (Tempel et al., 1983). Similarly, a learning insect will avoid a CS that was combined with an aversive US during training (Tully and Quinn, 1985). Conditioning is most effective when the onset of the CS precedes the presentation of the US and/or is presented at the same time. This type of conditioning is called “forward pairing” if the CS precedes the US or “simultaneous conditioning” if the CS is only present during the presentation of the US. If the CS is presented after the US, this is called “backward conditioning” (Mahoney and Ayres, 1976). It is used to train the insect to associate the absence of the CS with the US (Felsenberg et al., 2014).

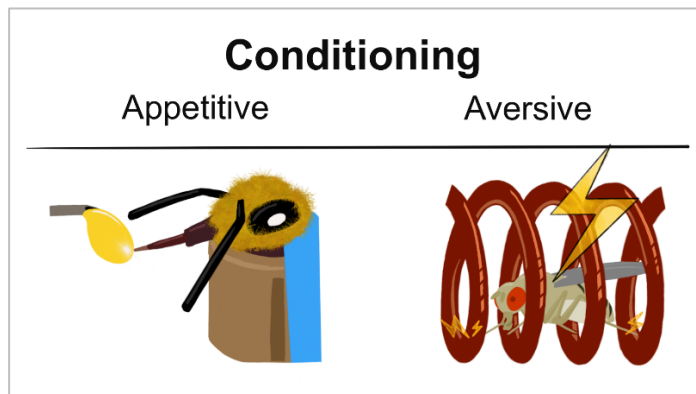


Figure 1: Training insects in the lab. Appetitive conditioning includes an unconditioned stimulus (US) that positively reinforces, aversive conditioning an US that negatively reinforces behavior. *Appetitive conditioning:* The honeybee (*Apis mellifera*) extends its proboscis to feed on the sugar reward. This behavior is called proboscis extension reflex (PER) and can be used as a proxy for learning. If the bee extends the proboscis in the presence of the conditioned stimulus (CS) after being trained with both US and CS, the CS is considered learned. *Aversive conditioning:* Fruit flies (*Drosophila melanogaster*) as well as other insects can be trained to avoid a CS that is presented together with an aversive US like an electric shock. Illustration adapted from Adam et al. (2022); © 2022 Adam, Hansson and Knaden.

In the past it was thought that insects need multiple (forward paired) learning experiences that are spaced apart in time in order to learn (long term) associations between CS and US (DeZazzo and Tully, 1995, Tully et al., 1994). This believe is, however, being challenged by more and more studies that show that insects can indeed learn fast and are able to learn associations in as little as one trial (Henaut et al., 2014, Huber and Knaden, 2018, Krashes and Waddell, 2008, Lee et al., 2020, Villar et al., 2020).

Insect Olfaction and Olfactory Learning

The world of insects is a world full of odors that are important in all areas of life of the insect. Odor information is exchanged during communication, is key for successful reproduction, plays an important role during foraging and navigation, and allows the insect to determine where to oviposit (Bisch-Knaden et al., 2018, Buehlmann et al., 2014, Buehlmann et al., 2015, d'Ettorre et al., 2017, Huber and Knaden, 2018, Landolt, 1997, Renou and Anton, 2020). Since odors play such a great role in the life of the insect, its olfactory system deserves a closer look: Odors are detected by fine structures called “sensilla”. These sensilla are mainly found on the antennae and maxillary palps (Carey and Carlson, 2011), but can also be present in other areas of the insect’s body such as the proboscis or the ovipositor (Haverkamp et al., 2016, Klinner et al., 2016). The sensilla contain olfactory sensory neurons (OSNs), also called olfactory receptor neurons (ORNs), which are equipped with olfactory receptors. Depending on which type of olfactory receptor the neurons express, they will respond to different types of volatile compounds (Masson and Mustaparta, 1990). Receptors can be narrowly tuned, that is the sensory neuron will only respond to one or few odors, or broadly tuned responding to multiple odors (Hallem and Carlson, 2006). When a sensillum of the antenna detects an odor, this odor information is sent from the periphery to the central nervous system of the insect (**Figure 2**).

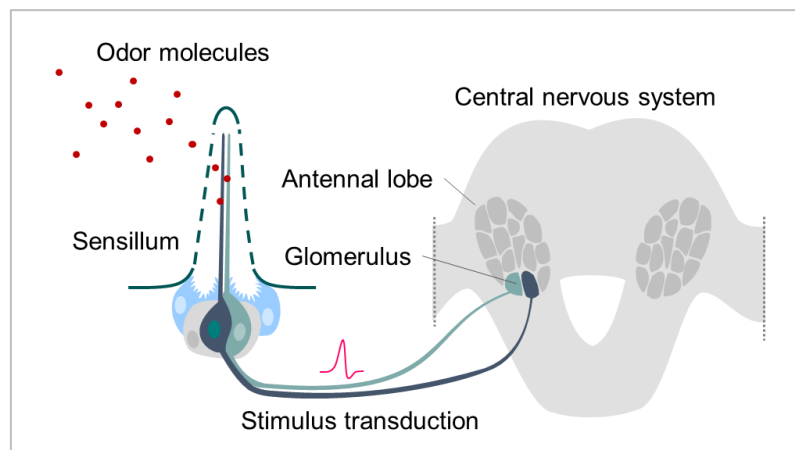


Figure 2: Odor perception in insects. The sensillum detects odor molecules in the environment. Upon detection, the odor information is sent to the central nervous system (CNS). The first relay center of the CNS is the antennal lobe (AL). It consists of small sphere-shaped structures called “glomeruli”. Illustration by Elisabeth Adam. Sensillum based on Kaissling (2019).

There, the OSNs connect to higher order neurons called projection neurons (PNs) and local interneurons (LNs) in the antennal lobe (AL) (Stocker et al., 1990). From the PNs, the information is sent further to higher brain centers named the mushroom bodies (MBs) and/or the lateral horn (LH) (Homberg et al., 1989) (**Figure 3**).

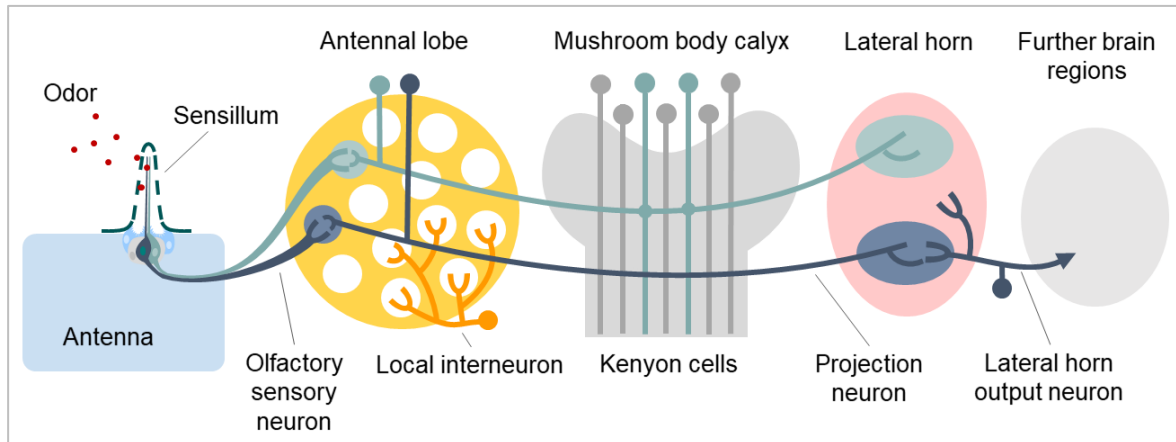


Figure 3: Schematic representation of the olfactory system of the insect. Odor information is sent from the antenna to the antennal lobe (AL) via olfactory sensory neurons (OSNs). Local interneurons (LNs) connect different glomeruli in the AL. From the AL, projection neurons (PNs) send the information to the mushroom body (MB) and/or lateral horn (LH). Some of the projection neurons (PNs) synapse with the MB neurons, also named Kenyon cells, at the dendritic zone of the MB, called the MB calyx. Others bypass the MB and directly connect to the LH. The information from the LH is then sent to other brain regions like the superior lateral protocerebrum (SLP) or back to the MB via lateral horn output neurons (LHONs). Illustration by Elisabeth Adam based on Kymre et al. (2021), Masse et al. (2009), and Zhao and McBride (2020).

These higher brain centers have two different functions. The MB is important for olfactory learning and memory, therefore, connections from PNs to the MB are highly variable and allow for plasticity. In contrast, the LH mainly processes odor information that involves innate rather than learned olfactory behavior. Connections between PNs and the LH are more stereotyped which is consistent with its less plastic function (Carey and Carlson, 2011). Further downstream, the cells of the MB, called Kenyon Cells (KCs), are connected to the mushroom body output neurons (MBONs) and dopaminergic neurons (DANs) (Aso et al., 2014). While MBONs regulate the approach or avoidance of a stimulus (Boto et al., 2020), DANs encode the valence of the US and are e.g. required for signaling the reinforcing property of a sugar reward (Liu et al., 2012, Mao and Davis, 2009). Innate as well as learned valences are integrated by a neuronal population called “convergence neurons” (CNs) downstream of the MBONs (Eschbach et al., 2021). The lateral horn also connects to further brain regions via lateral horn output neurons (LHON).

These neurons do not seem to drive motor behavior directly, but may rather connect to other neurons integrating memory and/or internal state (Dolan et al., 2019).

The structure of the insect's olfactory system already shows how interconnected olfaction and learning are. Although there are neuronal pathways encoding and driving innate olfactory behavior, a great percentage of the olfactory network is dedicated to odor learning. In terms of conditioning, an odor can be seen as CS and for example a nectar reward as US.

The Tobacco Hawkmoth

To investigate the different aspects of odor learning for this thesis, I was working with the tobacco hawkmoth (*Manduca sexta*). *M. sexta* is a crepuscular flower-visiting insect that has been well established as a model organism for olfactory perception and learning (Bisch-Knaden et al., 2018, Daly et al., 2001a, Daly et al., 2001b, Daly and Smith, 2000, Goyret et al., 2007, Hanson and Dethier, 1973, Howlett et al., 2012, Riffell et al., 2008, Tichenor et al., 1981, Yamamoto and Fraenkel, 1960). It relies on olfactory as well as visual cues during foraging (Raguso and Willis, 2002, Raguso and Willis, 2005) and switches to solely olfactory cues when light levels are low (Goyret and Yuan, 2015). When it comes to flower odors, adult *M. sexta* moths innately prefer night-blooming flowers such as *Datura wrightii*, *Ipomoea longifolia* and *Nicotiana sp.* with similar odor emission profiles (Riffell et al., 2013). However, what is important to note is that the moths are able to learn to feed on other flowers such as *Agave palmeri* (Asparagaceae) that are not innately attractive to them in the wild (Riffell et al., 2008, Riffell et al., 2013). This shows that *M. sexta* is able to learn to associate the flower odors of these plants (CS) with the nectar reward (US) they provide and that this capability is of ecological relevance. Recent work from our department has shown that *M. sexta* is not only able to perceive odors with the antennae like other insects, but also with two sensilla on the tip of its proboscis, that is with its tongue (Haverkamp et al., 2016). This of course raises the question whether these sensilla are similarly involved in flower odor learning like the antennae. I answer this research question in Manuscript I.

While adult tobacco hawkmoths mainly feed on the nectar of solanaceous plants (Alarcón et al., 2008) (**Figure 4A**), the larvae are herbivores and feed on their leaves (Yamamoto and Fraenkel, 1960). Since the larvae have to fend for themselves as soon as they hatch, choosing appropriate plant leaves for oviposition is of high importance for the female to ensure the survival of the offspring (**Figure 4B**).

M. sexta relies on olfactory cues to assess the quality of the host plant for oviposition. The volatile organic compound (VOC) profiles of undamaged and herbivore-damaged plants differ. This allows the moth to avoid plants that are already damaged by other *M. sexta* larvae in order to avoid feeding competition (Allmann et al., 2013, Reisenman et al., 2009, Zhang et al., 2019) or to specifically select plants that promise (olfactory) protection against parasitoid wasps (Zhang et al., 2022). Although the three-lined potato beetle (*Lema daturaphila*) stands in feeding competition with *M. sexta*, it deters the parasitoid wasp *Cotesia congregata*, one of *M. sexta*'s main enemies, when feeding on the same host plant as *M. sexta*. Therefore the moth prefers to oviposit on beetle-damaged plants in order to protect the offspring from the parasitoid (Zhang et al., 2022).

Female tobacco hawkmoths decide whether to oviposit on a host plant based on olfactory cues that are perceivable from a distance as well as through direct contact with the plant leaves (Yamamoto et al., 1969). Still, direct contact with the plant is not strictly necessary to induce egg-laying behavior since the odor of host plants is enough to not only induce directed flight behavior, but also curling of the abdomen – a sign that eggs are about to be deposited (Mechaber et al., 2002).

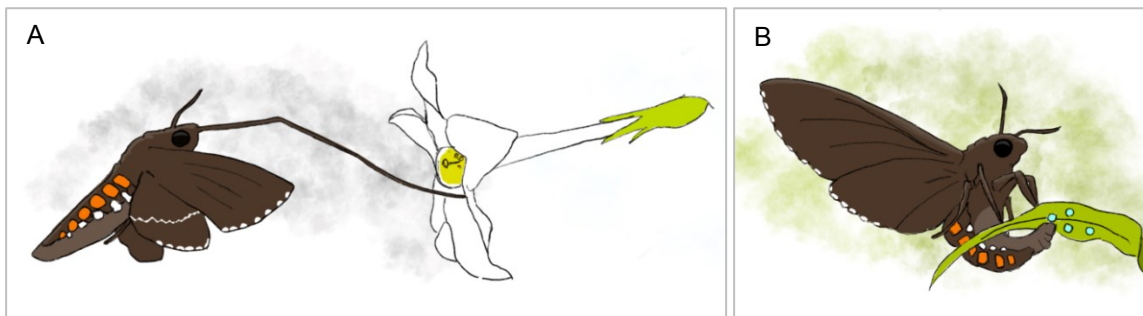


Figure 4: *Manduca sexta* olfactory behavior. A. *M. sexta* investigating a *Nicotiana sp.* flower. The hawkmoth uses its sense of smell to find flowers in the vast landscapes of its arid habitat. When finding a flower, it first investigates it with its proboscis. Only then it inserts the proboscis to feed from the nectar. B. *M. sexta* laying eggs on a host plant. The female hawkmoth first assesses the host plant odor from afar. Only if the plant odor passes the test, the moth will sit down and curl the abdomen to oviposit. Illustration by Elisabeth Adam.

In Manuscript II my colleague and I were interested in whether oviposition choices in *M. sexta* are influenced by previous experience. In the context of foraging, insects are known to learn rewarding flowers quickly in regard to sensory and motor memory. This is called “flower constancy” and helps the insect to reduce flower handling costs (Chittka et al., 1999, Waser, 1986). In Manuscript II we therefore asked whether learning is similarly involved in oviposition choices and whether *M. sexta* shows “oviposition constancy”.

Methodology: Behavioral Experiments in the Wind Tunnel

All experiments to assess moth olfactory behavior for this thesis were conducted in the wind tunnel of the Max Planck Institute for Chemical Ecology (**Figure 5**).

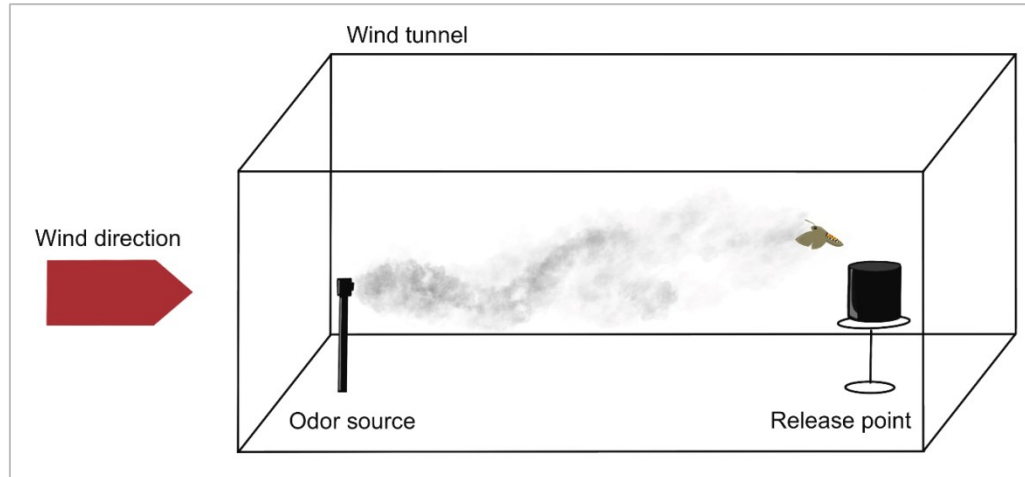


Figure 5: Wind tunnel set-up at the institute. The moths are released at the downwind end of the wind tunnel. An odor source at the upwind end of the wind tunnel provides an odor stimulus. Drawing not to scale. Illustration by Elisabeth Adam.

The wind tunnel allows to precisely control wind speed, temperature, humidity, and light levels in order to standardize experimental conditions. It therefore allows to simulate an as natural as possible foraging or oviposition situation in the laboratory. Moths have the innate behavior of flying upwind to track an odor. Therefore, releasing the moth on the downwind end of the wind tunnel lets the moth track (host plant) odors that are emitted by plants, testing- or training set-ups upwind. Moths were trained as well as tested individually.

For Manuscript I only male moths were used since this prevented unwanted oviposition during the foraging tasks. For Manuscript II female moths were needed since learning in the context of oviposition was the topic of this second manuscript.

Aim of This Thesis

There are three main aims of this thesis regarding insect olfactory learning:

The Context of Foraging

Olfactory sensilla are found in seemingly unusual places in *M. sexta* such as on the proboscis (Haverkamp et al., 2016) or the ovipositor (Klinner et al., 2016). Their functions are not clear and raise the question as to what they are used for. The first aim of this thesis was to elucidate whether the olfactory sensilla on the tip of the proboscis are involved in flower odor learning. This is the topic of Manuscript I. I hypothesized that they would play a role in odor learning since this could give an advantage for consecutive flower investigations in terms of flower handling.

The Context of Oviposition

The second aim of the thesis was to determine whether *M. sexta* shows learning during oviposition choices. This is the topic of Manuscript II. My colleague and I hypothesized that *M. sexta* would show oviposition constancy after experiencing a host plant to reduce handling and investigation times.

One Trial Learning

Both Manuscript I and II show that learning is possible after only one experience. This stands in stark contrast with previous beliefs that insects need multiple experiences that are spaced apart in time in order to learn. Therefore, I have conducted a comprehensive literature search to find out whether other authors have found similar fast learning abilities in other insects. I have summarized the current literature in a short review about one trial learning in insects. This is the topic of Manuscript III. The field is still quite small and studies are limited, but I hope that this review can spark the interest of more people to investigate the fantastic learning abilities of insects.

Manuscript I: Moths Sense but do not Learn Flower Odors With Their Proboscis During Flower Investigation

Elisabeth Adam, Bill S. Hansson*, Markus Knaden*[‡]

*Authors share senior authorship

[‡]Author for correspondence (mknaden@ice.mpg.de)

Journal of Experimental Biology

Published: September 13th; 2021

J Exp Biol, 224(17):jeb242780; doi: 10.1242/jeb.242780

In the first manuscript, I investigated whether the olfactory sensilla on the tip of the proboscis of the hawkmoth *Manduca sexta* are involved in learning flower odors. Previous work from our department had shown that the hawkmoth possesses olfactory sensilla on the tip of its proboscis. I wanted to know whether these sensilla play a role in learning while the moth is investigating possible food sources. However, contrary to the olfactory sensilla on the antennae of the moth, the sensilla on the tip of the proboscis do not seem to be involved in learning flower odors. I therefore suggest an innate function rather than a function connected to learning, such as the quality assessment of the flower.

Author contributions:

Built on an idea conceived by all authors.

Experimental design: E.A. (70 %), M.K., B.S.H.

Performed experiments: E.A. (100 %)

Data analyses: E.A. (90 %), M.K.

Wrote manuscript: EA (90 %), M.K., B.S.H.

Provided materials: B.S.H.

FORMULAR 1

Manuskript Nr. I

Titel des Manuskriptes: Moths Sense but do not Learn Flower Odors With Their Proboscis During Flower Investigation

Autoren: Elisabeth Adam, Bill S. Hansson, Markus Knaden

Bibliographische Informationen:

Adam, E., Hansson, B. S., & Knaden, M. (2021). Moths sense but do not learn flower odors with their proboscis during flower investigation. The Journal of Experimental Biology, 224(17), jeb242780.
<https://doi.org/10.1242/jeb.242780>

Der Kandidat / Die Kandidatin ist:

☒ Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: publiziert

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Adam, E.	70 %	90 %	100 %	90 %	-
Hansson, B.S.	15 %	-	-	5 %	100 %
Knaden, M.	15 %	10 %	-	5 %	-
Summe:	100 %	100 %	100 %	100 %	100 %

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in (Mitglied der Fakultät)

RESEARCH ARTICLE

Moths sense but do not learn flower odors with their proboscis during flower investigation

Elisabeth Adam, Bill S. Hansson* and Markus Knaden*[‡]

ABSTRACT

Insect pollinators, such as the tobacco hawkmoth *Manduca sexta*, are known for locating flowers and learning floral odors by using their antennae. A recent study revealed, however, that the tobacco hawkmoth additionally possesses olfactory sensilla at the tip of its proboscis. Here, we asked whether this second 'nose' of the hawkmoth is involved in odor learning, similar to the antennae. We first show that *M. sexta* foraging efficiency at *Nicotiana attenuata* flowers increases with experience. This raises the question whether olfactory learning with the proboscis plays a role during flower handling. By rewarding the moths at an artificial flower, we show that, although moths learn an odor easily when they perceive it with their antennae, experiencing the odor just with the proboscis is not sufficient for odor learning. Furthermore, experiencing the odor with the antennae during training does not affect the behavior of the moths when they later detect the learned odor with the proboscis only. Therefore, there seems to be no cross-talk between the antennae and proboscis, and information learnt by the antennae cannot be retrieved by the proboscis.

KEY WORDS: *Manduca sexta*, Hawkmoth, Insect olfaction, Flower handling, *Nicotiana attenuata*, Learning

INTRODUCTION

While foraging, flower-visiting insects have to maximize their energy gain while keeping their energy expenditure at a minimum (Pyke, 1978). This is especially crucial for hovering insects such as hawkmoths, as they are faced with considerable energy costs during flight (Bartholomew and Casey, 1978). Therefore, it is of great advantage to learn reliable resources and avoid flowers that produce no nectar or nectar of low quality, cheating the insect out of a profitable meal. Because primary attractants such as nectar and pollen are often concealed, insects have to rely on other, secondary attractants, e.g. color, shape, flower scent, taste or texture, to locate the floral rewards (Wester and Lunau, 2017). This is particularly important, since not every nectar is scented, which would be a honest signal for the pollinator (Raguso, 2004).

Moths use both flower scent and visual displays as secondary attractants to locate their host flowers (Raguso and Willis, 2002,

2005; Stockl and Kelber, 2019). Depending on their circadian rhythm, the agile hawkmoths are known to assign different relative importance to these stimuli (Balkenius et al., 2006). While the diurnal moth *Macroglossum stellatarum* predominantly reacts to visual stimuli, the nocturnal moth *Deilephila elpenor* is strongly attracted by olfactory stimuli (Balkenius et al., 2006). The crepuscular hawkmoth *Manduca sexta*, in contrast, uses both visual and olfactory cues to locate host flowers (Raguso and Willis, 2002, 2005) and only solely relies on olfactory cues under very dim light conditions (Goyret and Yuan, 2015).

In the wild, *M. sexta* is innately attracted to the floral scent of *Nicotiana attenuata* and *Datura wrightii*, and is known to pollinate flowers that share a similar chemical odor profile (Kessler and Baldwin, 2007; Riffell et al., 2013). Many individual odorants of these flower profiles are sufficient to induce foraging behavior in *M. sexta*, while other odorants are innately neutral (Bisch-Knaden et al., 2018). Yet, the hawkmoth can also learn to follow innately neutral odors of rewarding flowers when it is first lured to these flowers via visual cues (Cook et al., 2020; Riffell et al., 2008, 2013). This ability to associate novel odors with a reward has also been demonstrated in tethered moths, which exhibited a proboscis extension reflex after they had perceived an odor together with a sucrose reward (Daly et al., 2001b; Daly and Smith, 2000). However, in nature, moths not only have to locate the flower but, in a subsequent step, also need to be able to find the nectar within the flower (Goyret and Raguso, 2006). This is no easy feat, as the moth has to hover in front of the flower to access the nectar, a behavior that is energetically quite costly (Bartholomew and Casey, 1978). The involvement of additional sensory channels, including vision and mechanosensation, has been shown to enhance flower handling capabilities: although vision is important during both flower tracking and flower investigation, mechanosensation only seems to play an important role during flower handling itself (Farina et al., 1994; Goyret, 2010; Goyret and Kelber, 2011).

Recently, Haverkamp et al. (2016b) described newly discovered olfactory sensilla at the tip of the moth's proboscis. These sensilla house Orco-positive neurons that are able to detect and process odorants and respond to several of the same floral odorants that are also detected by the moth's antennae (Haverkamp et al., 2016b). The study revealed that this additional olfactory sense at the tip of the proboscis lets the moth investigate an innately attractive odor within the flower. Here, we asked whether the olfactory sensilla at the tip of the hawkmoth's proboscis could have an additional function in olfactory learning. As hawkmoths are known for their fast olfactory learning via their antennae (Cook et al., 2020; Riffell et al., 2008), we argued that learning new olfactory properties of the flower via the proboscis could give the moth an advantage in the identification and handling of consecutive flowers. This would be particularly true if there was cross-talk between the proboscis and antennae, as inserting the proboscis to smell the odor is energetically more costly (as a result of hovering and tracking the

Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, D-07745 Jena, Germany.

*Authors share senior authorship

[‡]Author for correspondence (mknaden@ice.mpg.de)

© E.A., 0000-0003-4382-8994; B.S.H., 0000-0002-4811-1223; M.K., 0000-0002-6710-1071

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Received 27 April 2021; Accepted 13 August 2021

flower) than recognizing the odor from afar. Therefore, we investigated whether hawkmoths are able to become more proficient in handling natural flowers that emit an appetitive odor over time and whether they use their sense of smell on the proboscis to learn flower cues. Also, by carefully modifying the test paradigms and working with an artificial flower that allowed us to confine odor to the proboscis only, we explored whether there is indeed cross-talk between the two ‘noses’ of the hawkmoth.

MATERIALS AND METHODS

Hawkmoth rearing

Manduca sexta (Linnaeus 1763) moths were reared and maintained at the Max Planck Institute for Chemical Ecology (MPI CE). Adult moths were kept in a flight cage (122×42×76 cm) for mating. A *N. attenuata* plant served as a substrate for oviposition, and a hummingbird feeder with artificial flowers provided a 10% sugar solution. Eggs were collected 3 times a week and transferred to small plastic boxes containing an artificial diet for the emerging larvae (artificial diet: 306 g agar, 959 g wheat germ, 932 g corn meal, 506 g soy flour, 499 g casein, 160 g salt mix, 240 g sugar, 33.3 g cholesterol, 80 g ascorbic acid, 40 g sorbic acid, 20 g methyl paraben, 60 ml linseed oil, 200 ml vitamin mix and 12 l water; vitamin mix: 2000 mg nicotinic acid, 1000 mg riboflavin, 467 mg thiamine, 467 mg pyridoxine, 467 mg folic acid, 40 mg biotin and 2 l water). On reaching 2nd instar, larvae were transferred to larger boxes; 5th instar larvae, which stopped feeding, were moved to wooden planks with pre-drilled holes for pupation. Shortly before hatching, male and female pupae were separated and put into egg cartons inside small folding insect cages. Hatched moths were kept in larger flight cages until experimentation. Eggs, larvae and pupating larvae were reared in a climate-controlled chamber at 26°C and 40% humidity and a light:dark cycle of 15 h:9 h. Pupae and adult moths were kept in separate climate chambers for male and female moths at 25°C and 60% humidity during light hours and 23°C and 70% humidity during dark hours on a light:dark cycle of 16.5 h:7.5 h. For all behavioral experiments, 3 day old naive male moths, which had neither fed nor encountered an (artificial) flower before, were used.

Wind tunnel experiments

All behavioral experiments were conducted in a wind tunnel with laminar air flow (wind tunnel size: 250×94×90 cm; wind speed – charcoal filtered air: 0.4 m s⁻¹; temperature: 25°C; humidity 70–75%). Side, top and front cameras were used to record the movement of the moth within the wind tunnel (Logitech HD Webcam C615; ELP HD Digital USB Camera; recording speeds: 30 frames s⁻¹). White and red light sources produced low visible light (photosynthetic active radiation, PAR: 0.27 µmol m⁻² s⁻¹). To allow recording of the moth at these low light settings, the infrared filters of the cameras were removed and an infrared light source (not visible to the moth) was used for illumination. Videos were recorded using Noldus Media Recorder 2.5.228 software (Noldus, Wageningen, The Netherlands) to allow the simultaneous recording of different camera angles.

The hawkmoths were kept in individual small mesh cages (14.5 cm diameter and 14.5 cm high) and acclimatized to the wind tunnel conditions in a habituation chamber for at least 1 h prior to testing. In all paradigms, the moths started from a small platform (height: 33.5 cm) positioned centrally at the downwind end of the wind tunnel, about 10 cm from the wind tunnel wall. They were gently nudged with a brush to initiate wing fanning (for 1–2 min). As soon as a moth started to fly, it was allowed to explore the wind

tunnel for 5 min. Trials ended after the time had expired or if the moth landed, as the animals generally did not resume flight for several minutes after landing.

Real flower investigation

To establish whether learning improves the moths’ success in handling natural flowers and create a baseline learning curve in our wind tunnel set-up, we first tested the moths using flowers of the wild tobacco plant *N. attenuata*. It is known that *M. sexta* improves its flower handling at artificial flowers over time (Goyret and Raguso, 2006). However, we wanted to confirm that this is also the case for natural flowers. As *M. sexta* is known as a major pollinator of *N. attenuata* (Kessler et al., 2010, 2015), and it has been shown that the odor emitted by these flowers increases foraging motivation in the hawkmoth even in the absence of nectar (Haverkamp et al., 2016b), flowers of this plant were chosen for the paradigm. Compared with large flowers of plants such as *D. wrightii* (in which the moth simply has to drop its body and proboscis), the tiny flowers of *N. attenuata* are more difficult to handle (Haverkamp et al., 2016a). Therefore, we expected that learning-based improved flower handling would become more obvious with these rather tricky flowers. A flower array containing eight *N. attenuata* flowers was positioned at the upwind end of the wind tunnel (Fig. 1A). We decided to use a flower array instead of the whole plant to standardize flower angle and to allow for better video recording of the flower manipulation. In the wild, the flowers of *N. attenuata* move through a 140 deg arc from a pendant position (daytime) to an erect position (night-time) to facilitate interaction with the hawkmoth (Haverkamp et al., 2019; Yon et al., 2016, 2017). Therefore, we mounted the disc containing the flowers at a slight upward angle to ease the insertion of the proboscis. The *N. attenuata* flowers contained their natural nectar volumes. No additional sugar water or artificial nectar was added, in order to create as natural a testing situation as possible. The flowers were kept in water-filled 1.5 ml Eppendorf tubes with a hole drilled in the lid to prevent flower desiccation during testing (Fig. 1B). During the trial, the moth could freely investigate all eight flowers with its proboscis. It was counted as ‘moth flown’ if it initiated flight during the trial without settling down immediately afterwards. Moths that touched a flower within the flower array at least once with their proboscis were counted as ‘investigated flower’. To find out whether the moths become more efficient in flower handling, their success at inserting the proboscis into the flower during consecutive flower visits was analyzed.

Artificial flower design and training apparatus

To be able to train the moths and let them experience an odor together with a reward, we designed an artificial flower (Fig. 2A) to visually attract the moths to our training apparatus (Fig. 2B). We created a rather large flower compared with *N. attenuata* in order to create a supernormal stimulus and visually attract the moths. This was necessary as hawkmoths are less likely to respond to odorless flowers than to flowers emitting odor (Goyret et al., 2007; Raguso and Willis, 2002, 2005). The moths were rewarded with sugar water – in the presence or absence of the given training odor – at this artificial flower. The flower consisted of two layers of laser cut acrylic sheet with five petals and a central opening (flower measurements: 5.5×5.5×3 mm; central opening diameter: 6 mm). A white back layer defined the outline of the flower; a glossy light blue front layer (without UV reflective properties) enhanced the attraction of the flower (Fig. 2A). We chose these materials as our pre-tests with artificial flowers made with different materials/colors/reflective

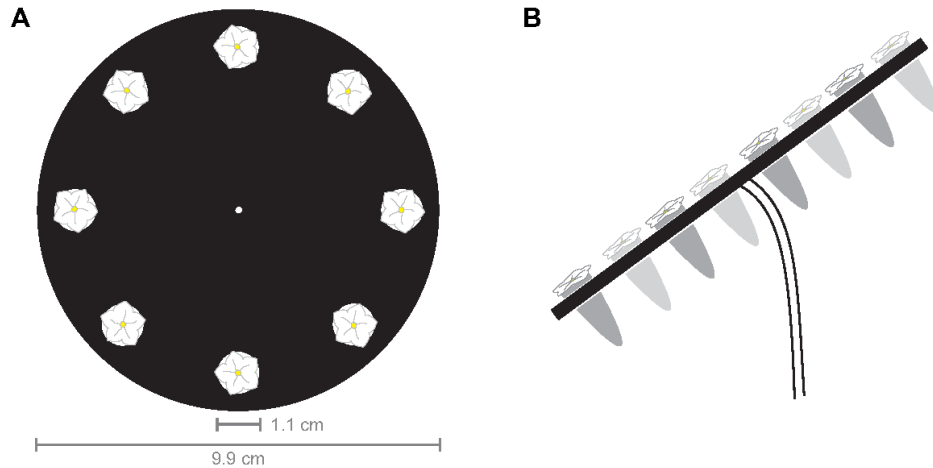


Fig. 1. Real flower array. (A) Black acrylic disc holding eight Eppendorf tubes with *Nicotiana attenuata* flowers that can be investigated by the moth. Tubes behind the disc provide water to prevent flower desiccation. (B) Side view of the flower array. The disc is held by a bendable rod to allow for an upward angle. Drawings not to scale.

properties had shown that hawkmoths have an innate preference for blue flowers with highly reflective properties outside the UV range, confirming the results of Goyret et al. (2008). Previous studies further showed that hawkmoths prefer radial patterns and that radial folds as well as a slight corolla curvature can improve flower handling efficiency (Campos et al., 2015; Goyret and Raguso, 2006;

Kelber, 2002). Therefore, we also added radial recesses leading to the center of the flower (radial recesses: 1 mm). The artificial flower was attached to an apparatus that enabled us to track the movement of the proboscis within the flower, so we could confirm that the moths reached the sugar reward. The apparatus (Fig. 2B) consisted of a top lid containing a video camera (Logitech Webcam 600;

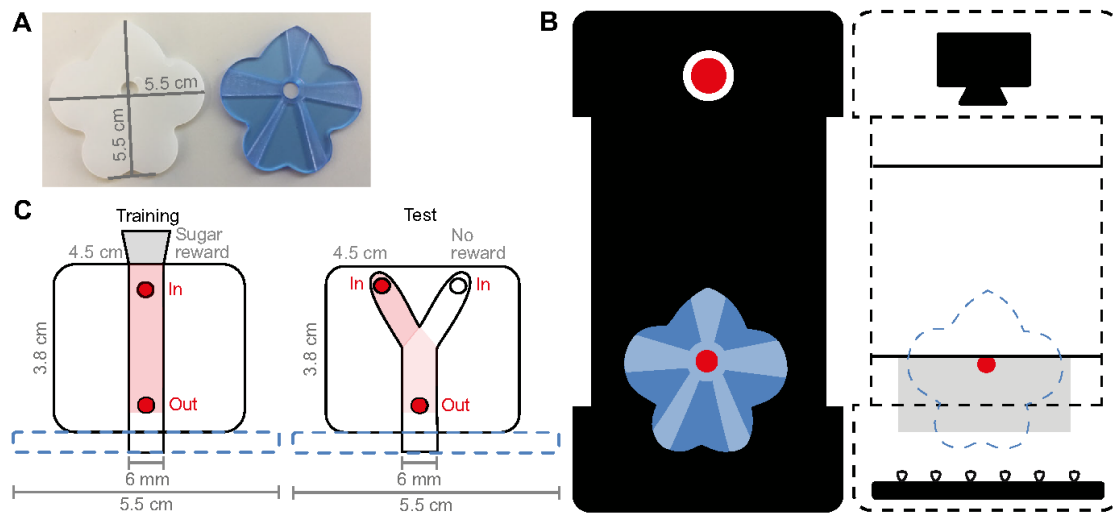


Fig. 2. Proboscis training apparatus. (A) Artificial flower. Left: white back layer to define flower shape. Right: light blue front layer with high reflective properties and radial recesses. (B) Training apparatus. Left: front view of the outside of the vertical apparatus. The artificial flower (blue) at the front of the training apparatus allows insertion of the proboscis. A clamp holding a small filter paper disc (red disc with white outline) for antennal odor stimulation can be attached to the front of the apparatus in such a way that the moth does not accidentally touch it while investigating the flower. Right: front view of the inside of the vertical apparatus. The grey rectangle indicates the space for the exchangeable elements (see C). A video camera (black) records the insertion of the proboscis from above. Infrared LEDs (small triangles) illuminate the proboscis training tube/Y-maze from below. Red circles indicate odor on the filter paper disc and/or in the proboscis training tube. (C) Top view of the exchangeable elements. Left: the proboscis can be inserted in a proboscis training tube through the artificial flower (blue outline). To exclude odor leakage out of the flower, odor is supplied through the valve labelled 'In' and removed by vacuum through the valve labelled 'Out'. The sugar reward (gray trapezium) is provided at the end of the proboscis training tube. Right: again, the proboscis can be inserted into the Y-maze through the artificial flower (blue outline). Odor and clean air (mineral oil control) (red and white circles) can be supplied through the valves labelled 'In' and removed through the valve labelled 'Out'. Drawings not to scale.

recording speed: 30 frames s⁻¹; resolution: 640×480 pixels), a middle part acting as a spacer/odor barrier and a bottom part containing an exchangeable element with either a linear proboscis training tube or a Y-maze (Fig. 2C). To be able to record the insertion of the proboscis, an infrared LED panel (Roschwege GmbH, LED puzzle piece, IR 850 nm) was added under the exchangeable element (Fig. 2B). The LEDs were powered at 11.6 V and 0.14 A (Manson switching mode power supply NRP 3630). An opening at the front of the training apparatus (diameter 6 mm) connected the artificial flower to the linear tube/Y-maze inside the apparatus. The training apparatus containing the proboscis training tube had an additional opening at the back (diameter 6 mm) to allow the attachment of a PCR tube with the sugar reward for conditioning (PCR tube: 200 µl, cut in half; sugar reward: 75 µl of 30% sucrose solution; sucrose: CAS 57-50-1). To facilitate the insertion of the proboscis, the training apparatus was mounted at a slight upward angle (+20 deg from the horizontal axis).

Odor choice paradigm

To investigate whether *M. sexta* moths are able to learn odors that they perceive with their proboscis, we first established the innate valence of the training odor in a choice experiment (Fig. 3A). In brief, individual moths were allowed to explore the wind tunnel and given a choice between two odor sources, one emitting clean air (mineral oil control), the other the training odor. We then measured the time the moths spent investigating both sources. We wanted to select an innately neutral odor (i.e. neither aversive nor attractive), to be able to show olfactory learning. Therefore, we chose linalool (+/-) (CAS 78-70-6), as it had previously been shown to be innately neutral in experiments investigating the antennal olfactory perception of the moth (Bisch-Knaden et al., 2018). Further, it can be perceived by the proboscis of the moth (Haverkamp et al., 2016b), which was key to investigate odor learning with the proboscis. It is also naturally found in the nectar of *N. attenuata* (Kessler and Baldwin, 2007), the flower we used for our real flower investigation paradigm. The innate valence test was then followed by a training step, where the training odor was combined with a sugar reward (Fig. 3B), and a re-test, to investigate whether this experience increased the valence of the training odorant (Fig. 3C).

All three steps were conducted with the same moth on the same day with at least 30 min between trials. During the test phases, the odorant as well as clean air (mineral oil control) were supplied via two odor valves without obvious visual cues, spaced 45 cm apart at the upwind end of the wind tunnel. The valves were positioned about 20 cm from the upwind tunnel wall and mounted on metal poles 40 cm above ground. Linalool [here and in all other experiments: linalool (+/-), CAS 78-70-6; dilution: 10⁻² in mineral oil, CAS 8042-47-5; loading volume: 10 µl] as well as solvent control (mineral oil, CAS 8042-47-5; loading volume: 10 µl) were pipetted onto small filter paper discs (diameter 1.3 cm, cut from a Rotilabo® Rundfilter) and placed in 50 ml glass bottles (SCHOTT, Duran®), so the headspace could be collected. Charcoal-filtered air was used to push the headspace through Teflon tubing (diameter 4 mm) to the odor valves at an air flow speed of 0.05 l min⁻¹. To exclude positional bias, the odor and control valve were switched between testing days. The time a moth spent at the odor valve minus the time spent at the control valve was used as a measurement of the odorant's valence. A significantly increased valence after the moth experienced the odor with the sugar reward (see below) would therefore indicate odor learning.

In the training paradigm, moths could perceive a training odor either with their antennae (and possibly their proboscis while

approaching the flower) or with the proboscis only, while being rewarded with sugar solution (Fig. 3B). For the first experiment – to allow odor perception with the antennae – a small filter paper disc (diameter 1.3 cm; cut from a Rotilabo® Rundfilter) was fixed with a metal clamp to the front of the training apparatus and was loaded with the training odor (Fig. 3B, upper panel). In the second experiment, we asked whether experiencing the training odor with the proboscis only is sufficient for odor learning. To prevent the moths' antennae from coming into contact with the training odor during the experiment, the odor was confined to the inside of the artificial flower using an air push (in; air flow speed: 0.05 l min⁻¹) and pull (out; air flow speed: 0.2 l min⁻¹) system (Fig. 3B, lower panel). Similarly, as with the odor valves, the training odor was pipetted onto a small filter paper disc and placed into a 50 ml glass bottle, so the headspace could be collected and pushed through the proboscis tube within the artificial flower. As before, a moth was counted as 'moths flown' if it initiated flight during the first test. Moths that touched the flower at least once with their proboscis were counted as moths that 'investigated flower'. In both experiments, a moth was considered trained when it had successfully foraged ('successful') at the artificial flower and had completely emptied the sugar reward. Only moths that met this training criterion were used for re-testing.

Proboscis Y-maze paradigm

In a second paradigm, we asked whether the experience with a training odor would affect the performance of the proboscis within the flower (Figs 4 and 5). In the first experiment (Fig. 4), we wanted to know whether experiencing an odor with the proboscis only would influence the handling of a flower, i.e. the choice of the Y-maze arm within the flower. Therefore, we confined the odor to the inside of the artificial flower during training as well as during the test. As this is quite an artificial test situation – flower odor can usually already be smelled with the antennae during the approach and probing of the flower (Haverkamp et al., 2019) – we decided to conduct a second experiment and additionally added odor on the outside of our artificial flower during training (Fig. 5). We reasoned that this situation would be ecologically more relevant as moths use their antennae to locate a flower and use their proboscis to investigate the flower in more detail. During the test, however, odor was again confined to the Y-maze within the artificial flower.

For ease of the experiments we decided to simplify the process by using a control and an experimental group of moths. So, instead of comparing the performance of a given moth before and after training, we compared the performance of trained moths with that of other naive moths. During the training step, the control group was exposed to filtered air only while the experimental group was exposed to the training odor linalool. Again, a sugar reward was offered as an unconditioned stimulus to allow odor learning. This time, the moths were subsequently tested with a proboscis Y-maze within the artificial flower (Figs 2C, 4B and 5B). One of the Y-maze arms contained the mineral oil solvent control, the other arm the training odor (Fig. 2C). By adapting the airflow within the Y-maze, we ensured that only one arm of the Y-maze carried the odor (air removal speed: 0.6 l min⁻¹). We recorded the first choice of the Y-maze arms and calculated the net time within the arm carrying the odor, i.e. (time in odor arm) – (time in control arm). Subsequently, we compared the control and experimental groups regarding their net time spent within the odor arm. As in the previous paradigm, a moth was counted as 'moths flown' if it initiated flight during the first test and as 'investigated flower' if it touched the flower at least

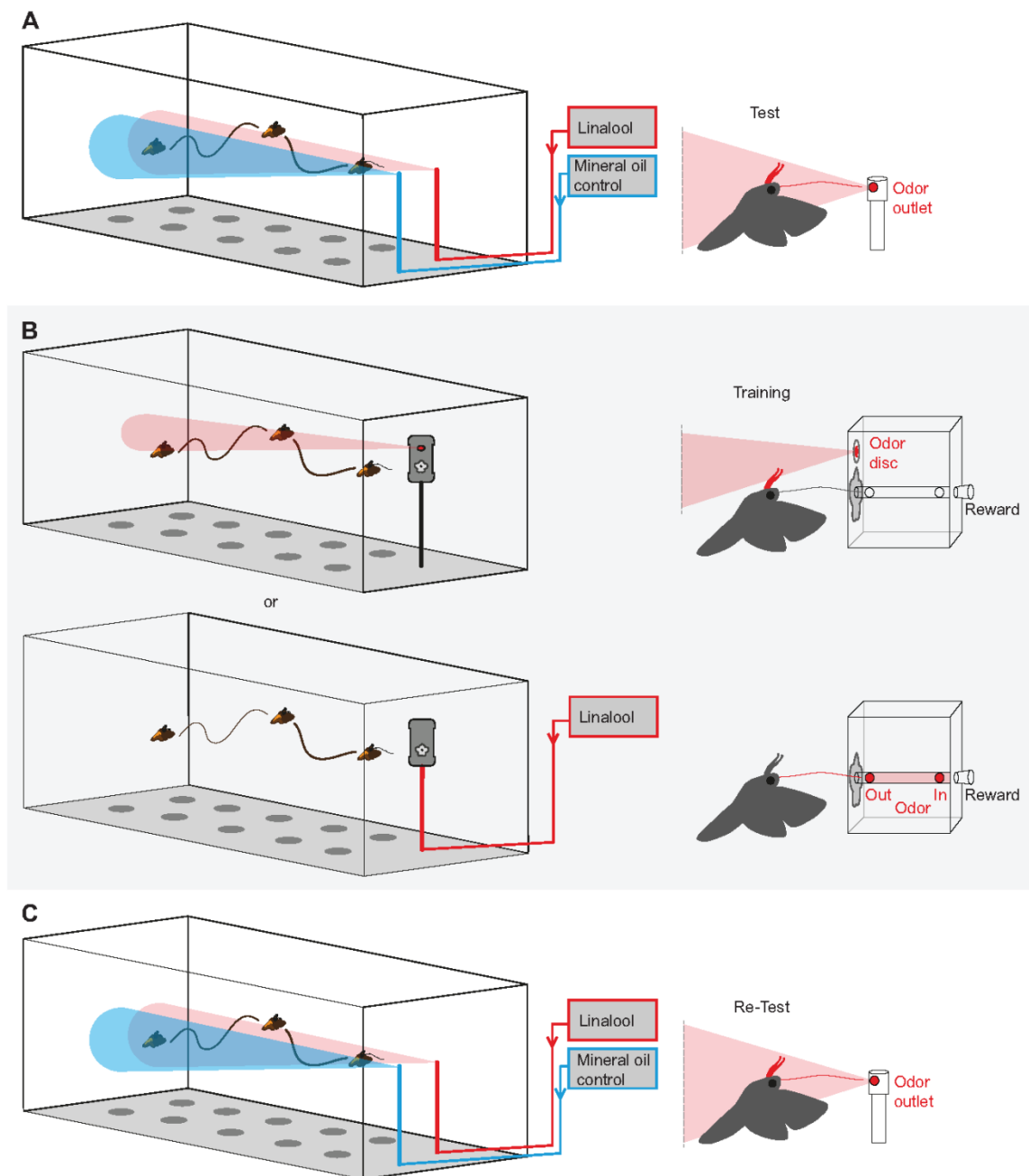


Fig. 3. *Manduca* odor choice paradigm. (A) Moths were tested for innate attraction to training odor in the wind tunnel. One odor valve supplied training odor (linalool, red), the other valve clean air (mineral oil control, blue). (B) During training, odor was either supplied to the antennae (top right) or restricted to the proboscis (bottom right) while a sugar reward was provided. (C) For the re-test, moths were again given a choice between the training odor (linalool) and clean air (mineral oil control). Drawings not to scale.

once with its proboscis. Only moths that had successfully foraged at the artificial flower and emptied the sugar reward ('successful') were used for testing.

Data analysis and statistics

The video data were analyzed manually using the software VLC media player 2.1.3 Rincewind. For the real flower investigation

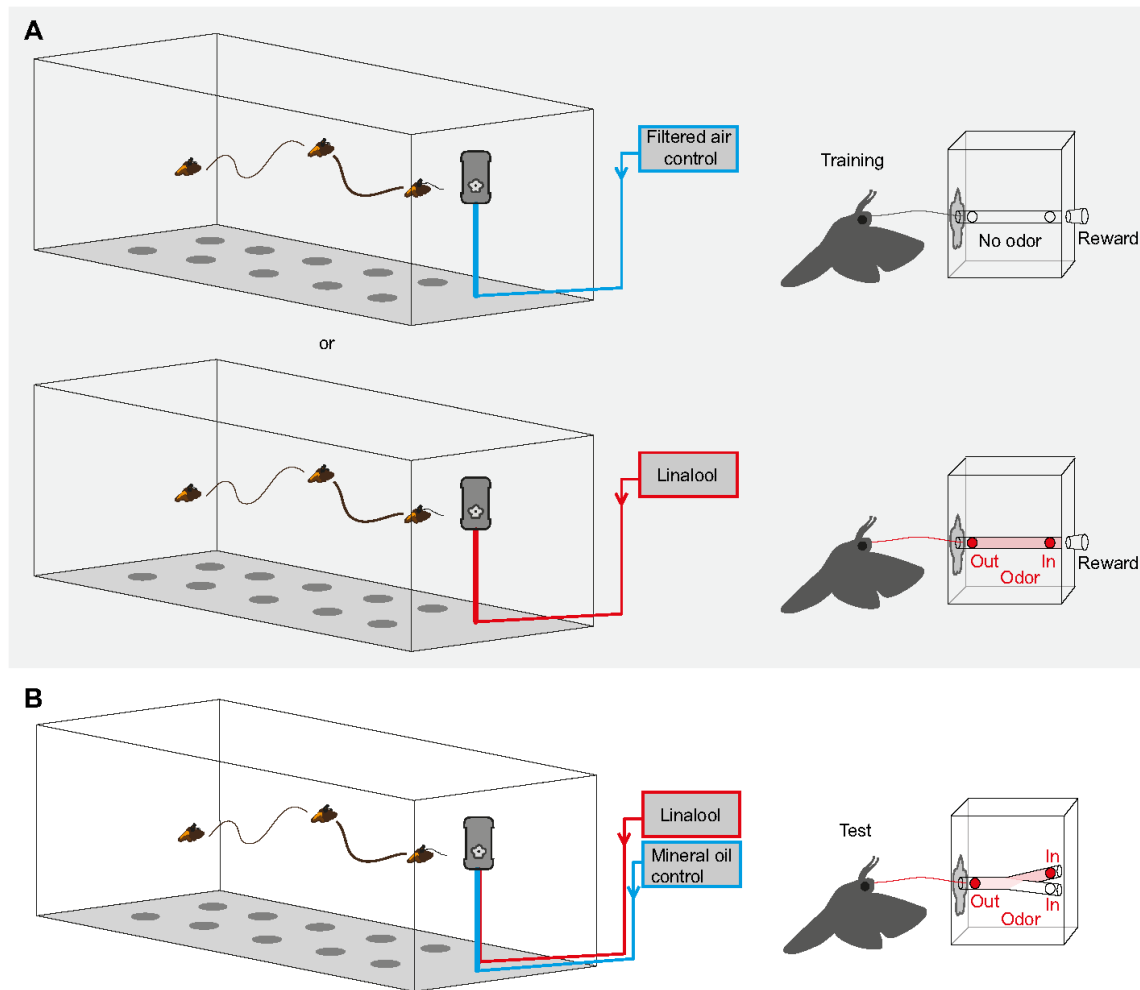


Fig. 4. *Manduca* proboscis Y-maze paradigm – proboscis learning only. (A) First, the moths were trained to investigate the artificial flower either in the absence (control group, top) or in the presence (experimental group, bottom) of the training odor. During training, the odor could only be perceived with the proboscis. (B) Moths were then tested with a proboscis Y-maze hidden within the artificial flower. One arm supplied the training odor (linalool), the other arm clean air (mineral oil control). Drawings not to scale.

paradigm, the time point and success of the first 20 flower contacts were recorded and used for analysis. Insertion of the proboscis into the flower corolla was counted as 'success', and investigation of the flower without subsequent insertion of the proboscis as 'no success'. In the case of success, the manipulation time until proboscis insertion (s) was recorded and the mean percentage success rate was calculated for the flower manipulations 1–5, 6–10, 11–15 and 16–20. For the odor choice paradigm time, the time spent at the control valve (s) and the time spent at the odor valve (s) were recorded before and after training. The net contact duration at the odor valve was calculated by subtracting the time spent at the control valve from the time spent at the odor valve. Similarly, the time spent in the control versus odor arm of the proboscis Y-maze was recorded and the net duration in the odor arm calculated for the proboscis Y-maze paradigm. Additionally, first choice and number of choices for each Y-maze arm were recorded for this paradigm and analyzed.

Statistical tests were performed using the statistics software GraphPad Prism version 7.05 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) or SPSS Statistics for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). Data were tested for normality using the Shapiro–Wilk normality test and further analyzed with an appropriate parametric or non-parametric test.

RESULTS

Hawkmoths learn how to manipulate flowers successfully with the proboscis

In order to investigate whether the moths' efficiency at natural flowers improves with experience and to create a learning baseline, we tested the moths using a *N. attenuata* flower array in the wind tunnel. Out of 107 'moths flown', a total of 29 moths explored the flower array and touched at least one flower with their proboscis

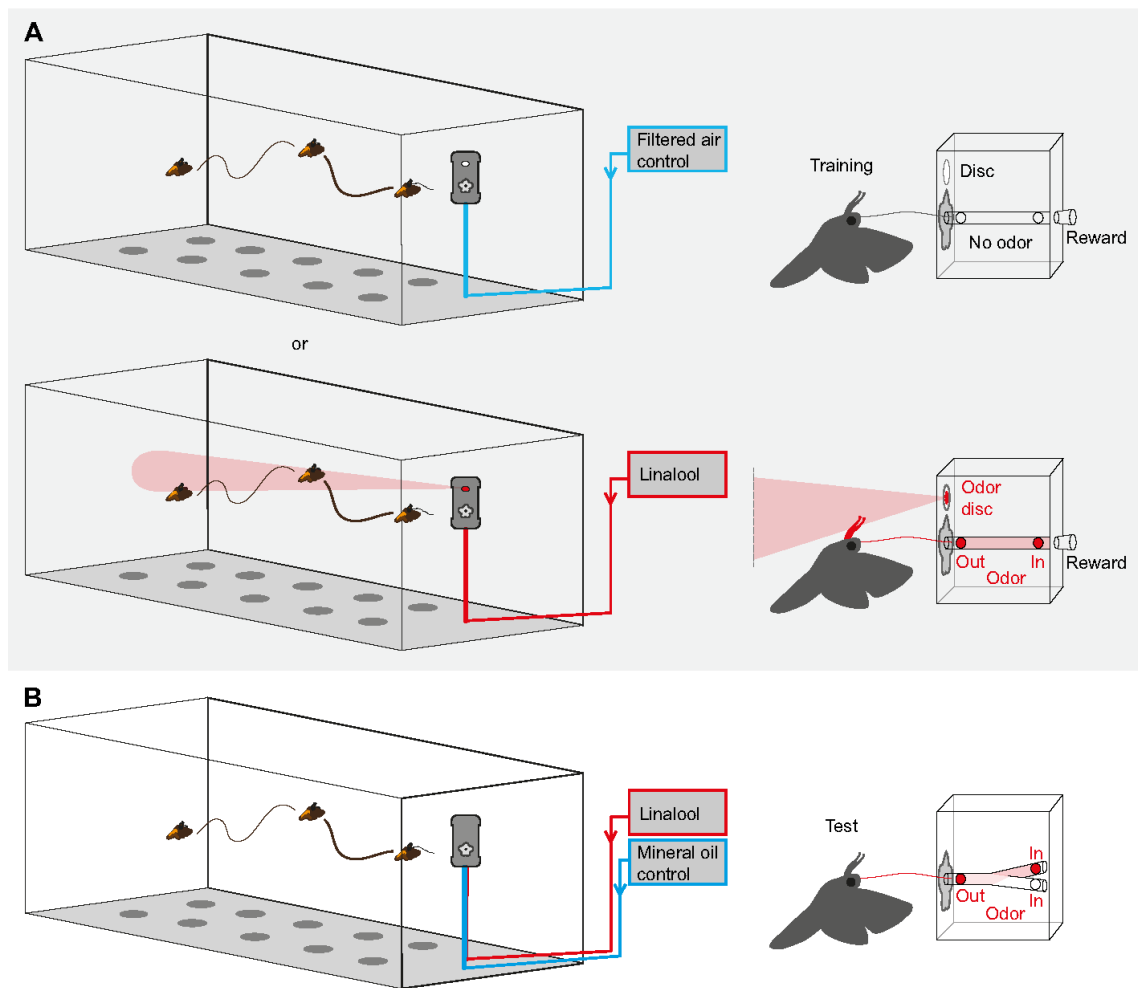


Fig. 5. *Manduca proboscis* Y-maze paradigm – proboscis and antennal learning. (A) Moths were trained to investigate the artificial flower either in the absence (control group, top) or in the presence (experimental group, bottom) of the training odor. During training, odor could be perceived with both the antennae and proboscis. (B) Moths were then tested with a proboscis Y-maze hidden within the artificial flower. As in the previous experiment, one arm supplied training odor (linalool) and the other arm clean air (mineral oil control). Drawings not to scale.

(‘investigated flower’). Of these, 31.0% had no success manipulating the flower, i.e. did not insert the proboscis into the flower opening; 51.7% had a success rate of $\geq 50\%$; and 20.7% had an overall success rate of $\geq 75\%$. Out of the 29 moths, 17 moths were highly motivated and had 20 (or more) flower contacts. When looking at the first five flower manipulations, the highly motivated animals exhibited a higher success rate than moths with fewer total flower contacts, suggesting that success increases the motivation to forage (Fig. 6A). Further analysis of moths with 20 flower contacts showed that they do learn to manipulate the flowers and become more successful over time (Fig. 6B). The shortest time of flower manipulation until proboscis insertion was less than 1 s, the maximum time 14 s. The average time before proboscis insertion was, however, 1 s. That means that the moths were generally quite quick to insert the proboscis into a non-moving *N. attenuata* flower at the given set-up.

Hawkmoths learn odorants with their antennae, but not with their proboscis

In the odor choice paradigm we tested whether *M. sexta* moths are able to learn odors with their antennae (moths flown: 46; investigated flower: 26; successful: 13; participated during re-test: 13) as well as their proboscis (moths flown: 28; investigated flower: 16; successful: 14; participated during re-test: 14) (Fig. 3). To assess whether our training odor (linalool) is innately neutral, we first established the valence of the training odor in a choice assay. As described previously by Bisch-Knaden et al. (2018), linalool was neither attractive nor aversive to naive moths as the moth spent the same amount of time at the two sources (Wilcoxon matched-pairs signed rank test, $n=27$, $P=0.125$). Hence, we were able to use it for our odor learning experiments. In a second step, i.e. the training step, the same moths were allowed to feed on sugar water from an artificial flower. During feeding, they could perceive linalool either

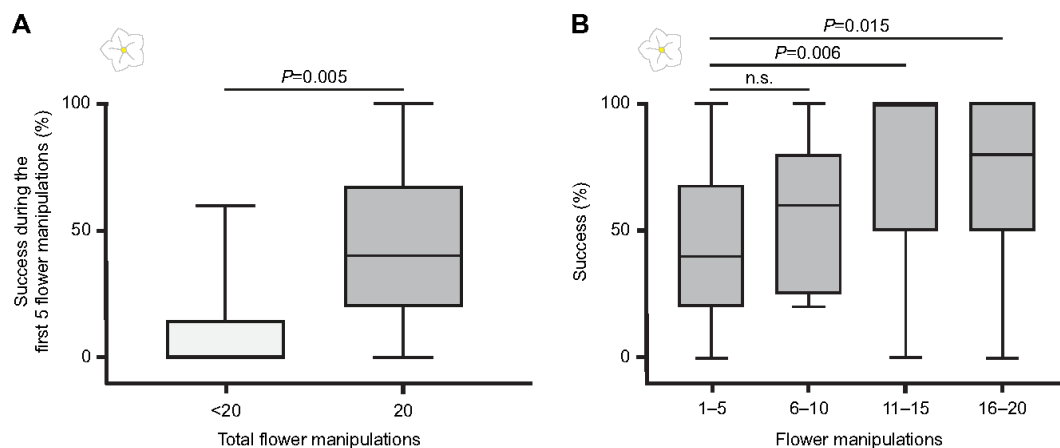


Fig. 6. Moths become more successful when investigating flowers. (A) During the first five flower contacts, success rates differed between moths that had few (<20: $n=8$) and many (≥ 20 : $n=17$) flower contacts. Moths that had higher success during the first five flower contacts tended to consecutively manipulate the flowers more often. Mann-Whitney U -test, $U=22.5$, $P=0.005$. (B) Feeding success increased with experience. When comparing the first five contacts with consecutive flower contacts, moths showed significantly higher success in later contacts (visits 11–15 and 16–20). Repeated measures ANOVA, $n=17$ moths, $F_{3,48}=6.136$, $P=0.001$. Planned contrasts, 1–5 versus 6–10: $F_{1,16}=2.537$, $P=0.131$; 1–5 versus 11–15: $F_{1,16}=10.112$, $P=0.006$; 1–5 versus 16–20: $F_{1,16}=7.509$, $P=0.015$. Box plots show the median, and first and third quartiles; whiskers show minimum and maximum values. White flower indicates real flower experiments.

by the antennae (and possibly the proboscis when approaching the flower) or by the proboscis only. After successful feeding, we again tested these moths for their preference for linalool in the aforementioned choice experiment. Moths that were allowed to perceive the odorant with their antennae during the training step readily learned the odorant and – in the subsequent test situation – spent significantly more time at the odor source than at the clean air source (mineral oil control) (Fig. 7A). In contrast, moths that were allowed to perceive the odorant during feeding with their proboscis only, later did not increase the time they spend at the odor source (Fig. 7B).

We next hypothesized that, although the odor experience via the proboscis does not affect the moths' approach towards an odor source, it could still affect the subsequent navigation of the proboscis within the flower. We therefore designed a paradigm where again the moths were first trained to an odor. However, this time, the moths did not have to approach the odor during flight in the test situation. Instead, the moths were attracted to an artificial flower by visual cues and were asked to pinpoint the odor within a proboscis Y-maze hidden in the artificial flower (Figs 4 and 5). As mentioned before, for reasons of simplification, we used a control and an experimental group of moths for this paradigm. In the first experiment, odor was confined within the artificial flower during training (control: moths flown: 68; investigated flower: 50; successful: 29; participated during test: 20; experiment: moths flown: 37; investigated flower: 26; successful: 22; participated during test: 20). In the subsequent test, both the control animals as well as the moths of the test group inserted their proboscis an equal amount of time in both arms of the Y-maze (Fig. 7C). Also, both groups of moths made their first choice randomly (control: binomial test, $n=20$, $P=0.824$; test: binomial test, $n=20$, $P=0.503$) and did not choose one of the arms more frequently than the other (control: Wilcoxon matched-pairs signed rank test, $n=20$, $P=0.850$; test: Wilcoxon matched-pairs signed rank test, $n=20$, $P=0.805$), indicating that the odorant was not learned via the proboscis. We concluded that experiencing a flower odor with the proboscis alone

affects neither the moths' later approach to the odor in flight nor their proboscis navigation within the flower.

At the same time, moths that smelled linalool with their antennae while being rewarded with sugar water, later targeted linalool in flight. We, therefore, asked whether the experience of the odor via the antennae might influence the moths' behavior with their proboscis, i.e. whether we would find information transfer from the antennae to the proboscis. In order to test this, we presented linalool to both the antennae and the proboscis, while the moth was feeding (Fig. 5A, bottom). For comparison, control moths were exposed to filtered air only during training (Fig. 5A, top). Afterwards, we again tested the navigation of the moths' proboscis in the Y-maze (control: moths flown: 81; investigated flower: 34; successful: 22; participated during test: 15; experiment: moths flown: 69; investigated flower: 32; successful: 19; participated during test: 15) (Fig. 5B). Both the control and the experimental group investigated the two arms of the Y-maze for a similar amount of time (Fig. 7D), made their first choice randomly (control: binomial test, $n=15$, $P>0.999$; test: binomial test, $n=15$, $P=0.607$), and did not choose one of the arms more frequently than the other (control: paired t -test, $n=15$, $t_{14}=0.078$, $P=0.939$; test: paired t -test, $n=15$, $t_{14}=0.345$, $P=0.735$). Evidently, although moths seemed to be able to learn the odor of a rewarding flower and to follow such a flower plume afterwards, the performance of the proboscis within the flower was not affected by learning.

DISCUSSION

Nectar-feeding insects such as bees, bumble bees, butterflies and moths are able to learn reliable food sources and return to flowers that promise high sugar rewards (Cnaani et al., 2006; Hill et al., 1997; Kandori and Yamaki, 2012; Kunze and Gumbert, 2001; Riffell et al., 2008). The specialization on one specific flower type can be of great advantage as it can save handling costs for the pollinator (Waser, 1986). Therefore, many insect species show flower constancy and keep visiting flowers of the same plant species that they have been successfully foraging at before

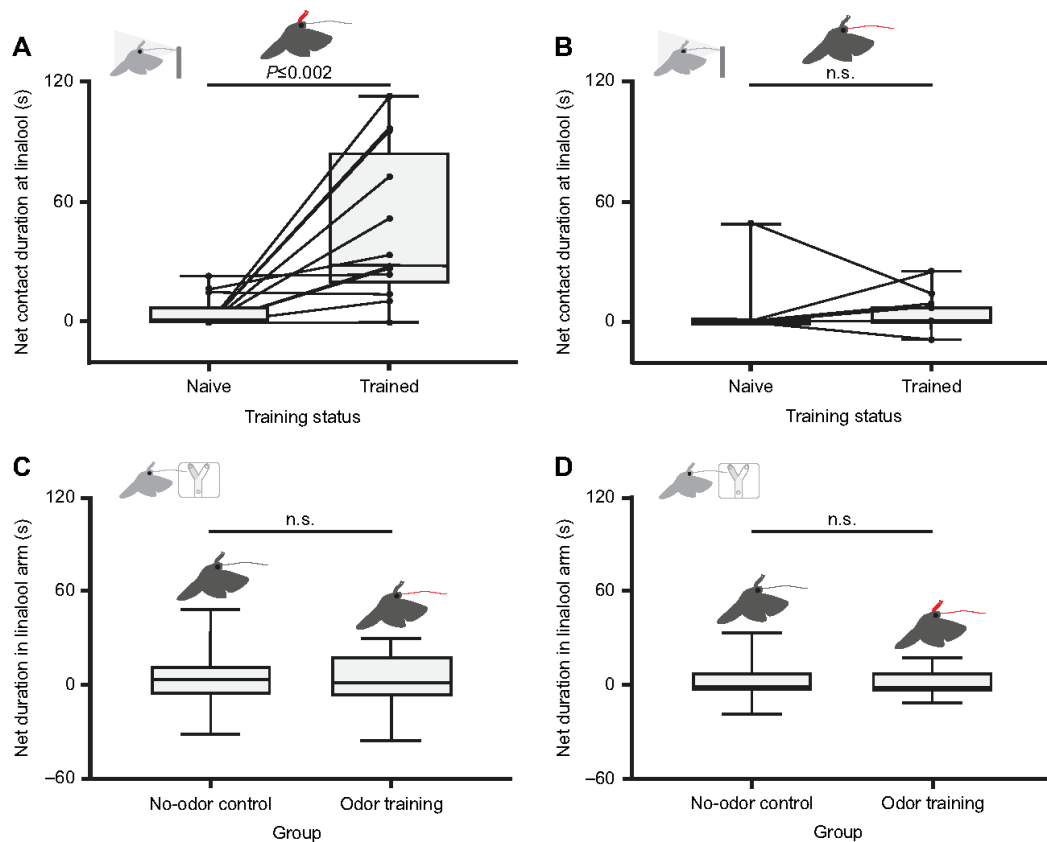


Fig. 7. Hawkmoths do not learn odors with their proboscis. (A) Linalool attraction before and after antennal training. Wilcoxon matched-pairs signed rank test, $n=13$, $P \leq 0.002$. (B) Linalool attraction before and after proboscis training. Wilcoxon matched-pairs signed rank test, $n=14$, $P > 0.999$. Moths experiencing linalool (+/–) with their proboscis (C) or antennae and proboscis (D) during training did not spend significantly more time in the linalool arm of the Y-maze than the control group. Unpaired t -test, C: $n=20$ per treatment, $t_{38}=0.329$, $P=0.744$; D: $n=15$ per treatment, $t_{28}=0.317$, $P=0.753$. Net contact duration at linalool (s): time spend at linalool (+/–) valve minus time spend at mineral oil control valve. Net duration in linalool arm (s): time spend in linalool (+/–) arm of Y-maze minus time spend in mineral oil control arm. Box plots show the median, and first and third quartiles; whiskers show minimum and maximum values; paired data of individual moths is visualized with connecting lines. Light gray moths: test paradigms; dark gray moths: training paradigms.

(Amaya Márquez, 2009; Cunningham et al., 1998; Goulson and Cory, 1993; Hill et al., 1997). Among other cues, flower odors play an important role in the establishment of flower constancy (Gegeer, 2005). So far, odor learning in moths has been assumed to function via the antennae only (Balkenius and Dacke, 2013; Cook et al., 2020; Cunningham et al., 2006, 2004; Daly et al., 2001a,b; Daly and Smith, 2000; Hartlieb, 1996; Ong and Stopfer, 2012; Riffell et al., 2008, 2013). However, hawkmoths (*M. sexta*) have olfactory sensilla not only on their antennae (i.e. their nose) but also on the tip of their proboscis, i.e. their tongue (Haverkamp et al., 2016b). Here, we asked whether hawkmoths become more efficient when feeding from flowers they have experienced before, and whether the olfactory sense on the proboscis is involved in this learning process.

To investigate whether hawkmoths learn from experience with a given flower type and create a baseline, we analyzed a moth's performance while consecutively feeding from several *N. attenuata* flowers provided in a flower array. Previous studies have shown that the feeding performance of *M. sexta* depends on the visited flower type or orientation (Campos et al., 2015; Haverkamp et al., 2019). However, we show that these hawkmoths, like butterflies

(Kandori and Ohsaki, 1996) and bumble bees (Chittka and Thomson, 1997; Laverty, 1980, 1994; Laverty and Plowright, 1988), indeed become more efficient when repeatedly foraging from one flower type that emits an ecologically relevant odor, i.e. *N. attenuata* flowers (Fig. 6B).

From experiments with (artificial) flowers (Chittka and Thomson, 1997; Goyret, 2010; Goyret and Kelber, 2011; Goyret and Raguso, 2006; Kandori and Ohsaki, 1996), we know that learning of mechano-sensory as well as visual cues is involved in such experience-based improved feeding efficiency. We wanted to know whether olfactory learning with the proboscis plays an additional role in this learning process. To be able to compare antennal with proboscis learning, we first tested the hawkmoths using an antennal learning paradigm. As described before by Balkenius and Balkenius (2010), moths were able to learn the rewarding odorant within one trial and chose the odor source with the rewarding odorant significantly more often than the mineral oil control (Fig. 7A). However, when the moths were able to smell the odorant only with their proboscis during training, they did not navigate towards the odor source emitting the rewarding odorant in

the consecutive test (Fig. 7B). We argued that this could either mean that the moths are not able to learn an odor with their proboscis or that moths are able to learn the odor with the proboscis, but do not use this information during the approach towards the flower. To find out whether hawkmoths use olfactory information learned with the proboscis while localizing nectar within the flower, we next tested the moths using a proboscis Y-maze. Again, we did not observe any learning. The moths spent equal amounts of time in the odor-emitting and the clean-air arm of the proboscis Y-maze after we trained them to the odorant (Fig. 7C). Given that encountering an odor with the proboscis alone is a rather artificial situation, we decided to do an additional experiment. In the wild, the moth can smell the odor of a flower even before inserting its proboscis in the flower corolla. That means that the odor can be considered a forward paired conditioned stimulus (CS) for the antennae as it will be present before the moth encounters the nectar (i.e. the unconditioned stimulus, US). In comparison, the proboscis might smell the odor for a much shorter time period when it is inserted into the flower. That means in terms of conditioning, this time span might be too short for the moth to create an association between odor (CS) and nectar (US) when using the proboscis to learn the CS. Still, would the moth be able to retrieve information via the proboscis that was acquired before by the antenna? In the desert ant *Cataglyphis fortis*, it was shown that the information about polarized light information acquired by one eye can later be retrieved by the other eye (Wehner and Müller, 1985). In dolphins such information transfer even happens between the visual and the acoustic sense (objects experienced by vision can later become recognized by echolocation and vice versa) (Pack and Herman, 1995). To test whether there is similar crosstalk between the moth's antennae and proboscis, we let the moth not only experience the odor via the proboscis inside the flower, but in addition already on the outside of the flower via the antennae (Fig. 5A). However, even this additional experience with the antennae did not affect the later performance of the moth's proboscis in the Y-maze (Fig. 7D). Hence, there does not seem to be any crosstalk between the antennae and proboscis taking place. We conclude that although *M. sexta* detects flower odors via both its antennae and the tip of the proboscis, only the antennae seem to be involved in the moth's olfactory learning. This might make sense from an ecological standpoint, as the antennae are used to find flowers at a distance. Hence, an early and correct decision, based on learning, might optimize energy gain. The proboscis 'nose', however, usually lies rolled up inside the moth head until the very last moment before flower contact. Although it stays unfurled during a foraging bout, a choice based on proboscis learning most likely would not yield a similar energy gain as hovering during the foraging bout is energetically costly.

So, what adaptive value could the olfactory sensilla on the tip of proboscis of the moth have? As Haverkamp et al. (2016b) suggested, these olfactory sensilla might be used to assess the quality of the flower. One could imagine a gustatory rather than an olfactory function as floral volatiles have been shown to be present in the nectar of some plant species (Kessler and Baldwin, 2007; Raguso, 2004). Also, appetitive volatile organic compounds in the nectar could increase the foraging motivation of the moth, similar to the way in which innately attractive benzyl acetone increases the foraging motivation in *M. sexta* when detected via the antennae (Haverkamp et al., 2016b). Contrary to our expectation, we did not find any increased foraging motivation when the moth perceived linalool (another ligand detected by the proboscis sensilla) with the proboscis. Single sensillum recordings by Haverkamp et al.

(2016b) had shown that the multiporous sensillum styloconica responds to benzyl acetone with 62.6 spikes s^{-1} and to linalool with 22.5 spikes s^{-1} . We therefore would have expected that there would be at least a baseline of attraction (maybe lower than that observed with benzyl acetone) and that learning would induce an increase in this baseline. However, we do not have any information on how the olfactory information of the proboscis is integrated in the brain of the moth and whether the difference in spike quantity is processed differently or translates in different ways to behavior. This of course raises the question of how the odor information of both 'noses' – the antennae and the proboscis – is processed in the moth's brain and how these pathways differ. It is known that odor information sensed by olfactory sensilla on the antenna of an insect is first processed by the antennal lobe and from there forwarded to higher brain centers such as the mushroom body and the lateral horn (Carey and Carlson, 2011). While the lateral horn codes for the innate valence of an odor, olfactory learning mainly takes place in the mushroom bodies (Carey and Carlson, 2011). It, therefore, will be interesting to test whether the olfactory information detected by the olfactory sensory neurons located on the tip of the proboscis is processed similarly or whether it is restricted to a local circuit innervating, for example, the subesophageal ganglion.

In summary, we suggest that the moth has two 'noses' dedicated to different tasks. The antennae are used for olfactory perception of the flower over distance. They are also important for learning to associate a flower odor with a nectar reward. The proboscis, however, seems to have a more limited function and might only be used for the assessment of flower quality.

Acknowledgements

We thank Hannah Rowland for visually characterizing the UV reflectance of the artificial flowers. We also thank Alexander Haverkamp, Richard Fandino and Jin Zhang for discussions concerning behavioral assays.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.A., B.S.H., M.K.; Methodology: E.A., M.K.; Formal analysis: E.A., M.K.; Investigation: E.A.; Data curation: E.A.; Writing - original draft: E.A.; Writing - review & editing: B.S.H., M.K.; Visualization: E.A.; Supervision: B.S.H., M.K.; Funding acquisition: B.S.H.

Funding

This study was funded by the Max-Planck-Gesellschaft. Open access funding provided by the Max Planck Society. Deposited in PMC for immediate release.

References

- Amaya Márquez, M. (2009). Floral constancy in bees: a revision of theories and a comparison with other pollinators. *Rev. Colomb. Entomol.* **35**, 206–216.
- Balkenius, A. and Balkenius, C. (2010). Behaviour towards an unpreferred colour: can green flowers attract foraging hawkmoths? *J. Exp. Biol.* **213**, 3257. doi:10.1242/jeb.045161
- Balkenius, A. and Dacke, M. (2013). Learning of multi-modal stimuli in hawkmoths. *PLoS ONE* **8**, e71137. doi:10.1371/journal.pone.0071137
- Balkenius, A., Rosén, W. and Kelber, A. (2006). The relative importance of olfaction and vision in a diurnal and a nocturnal hawkmoth. *J. Comp. Physiol. A* **192**, 431–437. doi:10.1007/s00359-005-0081-6
- Bartholomew, G. A. and Casey, T. M. (1978). Oxygen consumption of moths during rest, pre-flight warm-up, and flight in relation to body size and wing morphology. *J. Exp. Biol.* **76**, 11–25. doi:10.1242/jeb.76.1.11
- Bisch-Knaden, S., Dahake, A., Sachse, S., Knaden, M. and Hansson, B. S. (2018). Spatial representation of feeding and oviposition odors in the brain of a hawkmoth. *Cell Rep.* **22**, 2482–2492. doi:10.1016/j.celrep.2018.01.082
- Campos, E. O., Bradshaw, H. D., Jr. and Daniel, T. L. (2015). Shape matters: corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. *Funct. Ecol.* **29**, 462–468. doi:10.1111/1365-2435.12378
- Carey, A. F. and Carlson, J. R. (2011). Insect olfaction from model systems to disease control. *Proc. Natl. Acad. Sci. USA* **108**, 12987–12995. doi:10.1073/pnas.1103472108

- Chittka, L. and Thomson, J. D. (1997). Sensori-motor learning and its relevance for task specialization in bumble bees. *Behav. Ecol. Sociobiol.* **41**, 385-398. doi:10.1007/s002650050400
- Cnaani, J., Thomson, J. D. and Papaj, D. R. (2006). Flower choice and learning in foraging bumblebees: effects of variation in nectar volume and concentration. *Ethology* **112**, 278-285. doi:10.1111/j.1439-0310.2006.01174.x
- Cook, B., Haverkamp, A., Hansson, B. S., Roulston, T., Lerdau, M. and Knaden, M. (2020). Pollination in the anthropocene: a moth can learn ozone-altered floral blends. *J. Chem. Ecol.* **46**, 987-996. doi:10.1007/s10886-020-01211-4
- Cunningham, J. P., West, S. A. and Wright, D. J. (1998). Learning in the nectar foraging behaviour of *Helicoverpa armigera*. *Ecol. Entomol.* **23**, 363-369. doi:10.1046/j.1365-2311.1998.00149.x
- Cunningham, J. P., Moore, C. J., Zalucki, M. P. and West, S. A. (2004). Learning, odour preference and flower foraging in moths. *J. Exp. Biol.* **207**, 87-94. doi:10.1242/jeb.00733
- Cunningham, J. P., Moore, C. J., Zalucki, M. P. and Cribb, B. W. (2006). Insect odour perception: recognition of odour components by flower foraging moths. *Proc. R. Soc. B Biol. Sci.* **273**, 2035-2040. doi:10.1098/rspb.2006.3559
- Daly, K. C. and Smith, B. H. (2000). Associative olfactory learning in the moth *Manduca sexta*. *J. Exp. Biol.* **203**, 2025-2038. doi:10.1242/jeb.203.13.2025
- Daly, K. C., Chandra, S., Durtschi, M. L. and Smith, B. H. (2001a). The generalization of an olfactory-based conditioned response reveals unique but overlapping odour representations in the moth *Manduca sexta*. *J. Exp. Biol.* **204**, 3085-3095. doi:10.1242/jeb.204.17.3085
- Daly, K. C., Durtschi, M. L. and Smith, B. H. (2001b). Olfactory-based discrimination learning in the moth, *Manduca sexta*. *J. Insect Physiol.* **47**, 375-384. doi:10.1016/S0022-1910(00)00117-7
- Farina, W. M., Varjú, D. and Zhou, Y. (1994). The regulation of distance to dummy flowers during hovering flight in the hawk moth *Macroglossum stellatarum*. *J. Comp. Physiol. A* **174**, 239-247. doi:10.1007/BF00193790
- Gegebar, R. J. (2005). Multicomponent floral signals elicit selective foraging in bumblebees. *Naturwissenschaften* **92**, 269-271. doi:10.1007/s00114-005-0621-5
- Goulson, D. and Cory, J. S. (1993). Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecol. Entomol.* **18**, 315-320. doi:10.1111/j.1365-2311.1993.tb01107.x
- Goyret, J. (2010). Look and touch: multimodal sensory control of flower inspection movements in the nocturnal hawkmoth *Manduca sexta*. *J. Exp. Biol.* **213**, 3676-3682. doi:10.1242/jeb.045831
- Goyret, J. and Kelber, A. (2011). How does a diurnal hawkmoth find nectar? Differences in sensory control with a nocturnal relative. *Behav. Ecol.* **22**, 976-984. doi:10.1093/beheco/arr078
- Goyret, J. and Raguso, R. A. (2006). The role of mechanosensory input in flower handling efficiency and learning by *Manduca sexta*. *J. Exp. Biol.* **209**, 1585-1593. doi:10.1242/jeb.02169
- Goyret, J. and Yuan, M. L. (2015). Influence of ambient illumination on the use of olfactory and visual signals by a nocturnal hawkmoth during close-range foraging. *Integr. Comp. Biol.* **55**, 486-494. doi:10.1093/icb/icv009
- Goyret, J., Markwell, P. M. and Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *J. Exp. Biol.* **210**, 1398-1405. doi:10.1242/jeb.02752
- Goyret, J., Pfaff, M., Raguso, R. A. and Kelber, A. (2008). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* **95**, 569-576. doi:10.1007/s00114-008-0350-7
- Hartlieb, E. (1996). Olfactory conditioning in the moth *Heliothis virescens*. *Naturwissenschaften* **83**, 87-88.
- Haverkamp, A., Bing, J., Badeke, E., Hansson, B. S. and Knaden, M. (2016a). Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. *Nat. Commun.* **7**, 11644. doi:10.1038/ncomms11644
- Haverkamp, A., Yon, F., Keese, I. W., Mißbach, C., Koenig, C., Hansson, B. S., Baldwin, I. T., Knaden, M. and Kessler, D. (2016b). Hawkmoths evaluate scenting flowers with the tip of their proboscis. *eLife* **5**, e15039. doi:10.7554/eLife.15039
- Haverkamp, A., Li, X., Hansson, B. S., Baldwin, I. T., Knaden, M. and Yon, F. (2019). Flower movement balances pollinator needs and pollen protection. *Ecology* **100**, e02553. doi:10.1002/ecy.2553
- Hill, P. S. M., Wells, P. H. and Wells, H. (1997). Spontaneous flower constancy and learning in honey bees as a function of colour. *Anim. Behav.* **54**, 615-627. doi:10.1006/anbe.1996.0467
- Kandori, I. and Ohsaki, N. (1996). The learning abilities of the white cabbage butterfly, *Pieris rapae*, foraging for flowers. *Res. Popul. Ecol.* **38**, 111-117. doi:10.1007/BF02514977
- Kandori, I. and Yamaki, T. (2012). Reward and non-reward learning of flower colours in the butterfly *Byasa alcinous* (Lepidoptera: Papilionidae). *Naturwissenschaften* **99**, 705-713. doi:10.1007/s00114-012-0952-y
- Kelber, A. (2002). Pattern discrimination in a hawkmoth: innate preferences, learning performance and ecology. *Proc. R. Soc. B Biol. Sci.* **269**, 2573-2577. doi:10.1098/rspb.2002.2201
- Kessler, D. and Baldwin, I. T. (2007). Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *Plant J.* **49**, 840-854. doi:10.1111/j.1365-3113X.2006.02995.x
- Kessler, D., Diezel, C. and Baldwin, I. T. (2010). Changing pollinators as a means of escaping herbivores. *Curr. Biol.* **20**, 237-242. doi:10.1016/j.cub.2009.11.071
- Kessler, D., Kallenbach, M., Diezel, C., Rothe, E., Murdock, M. and Baldwin, I. T. (2015). How scent and nectar influence floral antagonists and mutualists. *eLife* **4**, e07641. doi:10.7554/eLife.07641
- Kunze, J. and Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav. Ecol.* **12**, 447-456. doi:10.1093/beheco/12.4.447
- Lavery, T. M. (1980). The flower-visiting behaviour of bumble bees: floral complexity and learning. *Can. J. Zool.* **58**, 1324-1335. doi:10.1139/z80-184
- Lavery, T. M. (1994). Bumble bee learning and flower morphology. *Anim. Behav.* **47**, 531-545. doi:10.1006/anbe.1994.1077
- Lavery, T. M. and Plowright, R. C. (1988). Flower handling by bumblebees: a comparison of specialists and generalists. *Anim. Behav.* **36**, 733-740. doi:10.1016/S0003-3472(88)80156-8
- Ong, R. C. and Stopfer, M. (2012). Peripheral and central olfactory tuning in a moth. *Chem. Senses* **37**, 455-461. doi:10.1093/chemse/bjr127
- Pack, A. A. and Herman, L. M. (1995). Sensory integration in the bottlenosed dolphin: immediate recognition of complex shapes across the senses of echolocation and vision. *J. Acoust. Soc. Am.* **98**, 722-733. doi:10.1121/1.413566
- Pyke, G. H. (1978). Optimal foraging in bumblebees and coevolution with their plants. *Oecologia* **36**, 281-293. doi:10.1007/BF00348054
- Raguso, R. A. (2004). Why are some floral nectars scented? *Ecology* **85**, 1486-1494. doi:10.1890/03-0410
- Raguso, R. A. and Willis, M. A. (2002). Synergy between visual and olfactory cues in nectar feeding by naïve hawkmoths, *Manduca sexta*. *Anim. Behav.* **64**, 685-695. doi:10.1006/anbe.2002.4010
- Raguso, R. A. and Willis, M. A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Anim. Behav.* **69**, 407-418. doi:10.1016/j.anbehav.2004.04.015
- Riffell, J. A., Alarcon, R., Abrell, L., Davidowitz, G., Bronstein, J. L. and Hildebrand, J. G. (2008). Behavioral consequences of innate preferences and olfactory learning in hawkmoth-flower interactions. *Proc. Natl. Acad. Sci. USA* **105**, 3404-3409. doi:10.1073/pnas.0709811105
- Riffell, J. A., Lei, H., Abrell, L. and Hildebrand, J. G. (2013). Neural basis of a pollinator's buffet: olfactory specialization and learning in *Manduca sexta*. *Science* **339**, 200-204. doi:10.1126/science.1225483
- Stöckl, A. L. and Kelber, A. (2019). Fuelling on the wing: sensory ecology of hawkmoth foraging. *J. Comp. Physiol. A* **205**, 399-413. doi:10.1007/s00359-019-01328-2
- Waser, N. M. (1986). Flower constancy: definition, cause, and measurement. *Am. Nat.* **127**, 593-603. doi:10.1086/284507
- Wehner, R. and Müller, M. (1985). Does interocular transfer occur in visual navigation by ants? *Nature* **315**, 228-229. doi:10.1038/315228a0
- Wester, P. and Lunau, K. (2017). Plant-pollinator communication. In *How Plants Communicate with their Biotic Environment*, Vol. 82 (ed. G. Becard), pp. 225-257. New York: Academic Press.
- Yon, F., Joo, Y., Cortés Llorca, L., Rothe, E., Baldwin, I. T. and Kim, S.-G. (2016). Silencing *Nicotiana attenuata* LHY and ZTL alters circadian rhythms in flowers. *New Phytol.* **209**, 1058-1066. doi:10.1111/nph.13681
- Yon, F., Kessler, D., Joo, Y., Cortés Llorca, L., Kim, S.-G. and Baldwin, I. T. (2017). Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*. *J. Integr. Plant Biol.* **59**, 180-189. doi:10.1111/jipb.12511

Manuscript II: Host Plant Constancy in Ovipositing *Manduca sexta*

Nandita Nataraj, Elisabeth Adam, Bill S. Hansson*, Markus Knaden**‡

*Authors share senior authorship

‡Author for correspondence (mknaden@ice.mpg.de)

Journal of Chemical Ecology

Published: September 21st, 2021

J Chem Ecol, 47(12):1042-1048; doi: 10.1007/s10886-021-01309-3

Flower visiting insects profit from repeatedly visiting the same flower species while foraging since this reduces handling costs and saves energy. The flowering plant benefits from this “flower constancy” as well since it ensures pollen transfer within plant species. In this second manuscript my colleague and I investigated whether hawkmoths (*Manduca sexta*) also show “oviposition constancy” and develop a preference for a plant species they have previously oviposited on. We could show that the oviposition experience indeed leads to a preference towards the plant species the hawkmoth has oviposited on before. Further, we could show that one successful encounter with a plant leaf is enough to form long term memories (LTMs) and to influence the moth’s preference.

Author contributions:

Built on an idea conceived by all authors.

Experimental design: N.N., E.A. (20 %), M.K., B.S.H.

Performed experiments: N.N., E.A. (20 %)

Data analyses: N.N., E.A. (30 %), M.K.

Wrote manuscript: N.N., EA (20 %), M.K., B.S.H.

Provided materials: B.S.H.

FORMULAR 1

Manuskript Nr. II

Titel des Manuskriptes: Host Plant Constancy in Ovipositing *Manduca sexta*

Autoren: Nandita Nataraj, Elisabeth Adam, Bill S. Hansson, Markus Knaden

Bibliographische Informationen:

Nataraj, N., Adam, E., Hansson, B. S., & Knaden, M. (2021). Host plant constancy in ovipositing *Manduca sexta*. *Journal of Chemical Ecology*, 47(12), 1042–1048. <https://doi.org/10.1007/s10886-021-01309-3>

Der Kandidat / Die Kandidatin ist:

☐ Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☒ Koautor/-in.

Status: publiziert

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Nataraj, N.	60 %	60 %	80 %	60 %	-
Adam, E.	20 %	30 %	20 %	20 %	-
Hansson, B.S.	10 %	-	-	10 %	100 %
Knaden, M.	10 %	10 %	-	10 %	-
Summe:	100 %	100 %	100 %	100 %	100 %

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in (Mitglied der Fakultät)



Host Plant Constancy in Ovipositing *Manduca sexta*

Nandita Nataraj¹ · Elisabeth Adam¹ · Bill S. Hansson¹ · Markus Knaden¹

Received: 24 May 2021 / Revised: 20 August 2021 / Accepted: 26 August 2021 / Published online: 21 September 2021
© The Author(s) 2021

Abstract

Many pollinating insects exhibit flower constancy, i.e. they target flower species they have already experienced and fed from. While the insects might profit from reduced handling costs when revisiting similar flowers, flower constancy, in addition, is of benefit for the plants as it guarantees pollen transfer to conspecifics. Here we investigate whether the previous experience of an insect can also result in oviposition constancy, i.e. whether ovipositing on a given plant species will drive future oviposition preference in a female insect. We show that female hawkmoths (*Manduca sexta*), after having oviposited on a given plant species only once, indeed will prefer this plant in future oviposition choices. As oviposition preference is even affected 24 h after the moth has oviposited on a given plant, long term memory seems to be involved in this oviposition constancy. Our data furthermore suggest that, as shown for flower constancy, ovipositing moths increase their handling efficiency by targeting those host plants they have already experienced.

Keywords Moth · Behavior · Host plant · Oviposition · *Nicotiana attenuata* · *Datura wrightii*

Introduction

Pollinators play an important role in maintaining the terrestrial ecosystem with more than 80% of flowering plant species relying on insect pollination (Ollerton et al. 2011). At the same time, herbivore insects and/or their offspring can cause huge damage to their host plants (Delaney et al. 2001). Therefore, plants should advertise themselves to potential insect pollinators, while hiding from insect herbivores. The relationship of the tobacco hawkmoth *Manduca sexta* and its host plants *Datura wrightii* and *Nicotiana attenuata* is specifically interesting as the moth is one of the most important pollinators of these plants, while its caterpillars can cause severe damage both on *Datura* and *Nicotiana* (Zhang et al. 2019).

The mutualistic relationship between plants and their pollinators often has resulted in a so-called flower constancy, where pollinators like bees (Gruter and Ratnieks 2011) or butterflies (Goulson et al. 1997) learn features of flowers

they have successfully foraged from and afterward specifically target similar flowers during their foraging flights. While the insect might profit from reduced handling costs when revisiting similar flowers, the plants profit from the guaranteed pollen transfer to conspecifics (Waser 1986).

Foraging tobacco hawkmoths exhibit an innate preference for specific flowers (Haverkamp et al. 2016) and floral odors (Bisch-Knaden et al. 2018). Such preferences for certain volatiles potentially enable pollinators to rapidly detect valuable floral resources without prior sampling. However, pollinators also show accurate learning abilities for those compounds reliably associated with a flower reward (Knauer and Schiestl 2015). This behavior might enable pollinators to switch between flower species, as soon as the most preferred flowers are not available anymore and are a prerequisite for the above mentioned flower constancy.

Indeed, also the tobacco hawkmoths' flower preference can be altered by learning. Having successfully foraged from a flower only once, the moths will target the flower's odor plume again, even if it was not innately attractive to the insect (Cook et al. 2020; Riffell et al. 2008).

Here we ask, whether ovipositing hawkmoths exhibit an innate preference for one of their host plants, *Datura* or *Nicotiana*, and whether previous oviposition experience with one of these plants results in a changed preference. We thus

Bill S. Hansson and Markus Knaden share senior authorship

✉ Markus Knaden
mknaden@ice.mpg.de

¹ Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

investigate, whether the moths exhibit any kind of learned oviposition constancy.

We find, that naïve moths slightly prefer to oviposit on *Datura* rather than on *Nicotiana*, when they are offered individual leaves of both plants. However, we furthermore show, that a single oviposition experience with either *Datura* or *Nicotiana* is sufficient to strongly tune the moths' preference towards the same plant, and at least for *Datura* moths can remember this experience even 24 h later.

To our knowledge, a similarly fast and long lasting learning of oviposition cues so far has been reported in parasitoid wasps only (Smid et al. 2007).

Methods and Materials

Insect rearing: All animals originate from eggs collected in Utah (DI Ranch, Santa Clara, UT, USA) in 2009 and since then were reared at the Max Planck Institute for Chemical Ecology, Jena, Germany. Female moths were allowed to oviposit on *Datura* plants. Eggs were collected immediately after oviposition and larvae were subsequently maintained on an artificial diet at 40% relative humidity and 26 °C with a light: dark cycle of 15:9. Fifth-instar caterpillars were individualized for pupation and left till 1 week before adults eclosed from the pupae at the same climate conditions. Pupae were sexed, and male and female pupae were transferred to separated flight cages with a light: dark cycle of 16.5:7.5, 60% relative humidity, and 25 °C during the light phase, and 60% relative humidity and 20 °C during the dark phase. Two-day-old female and male hawkmoths were kept for mating for 24 h. After roughly 6 h moths that did not mate were removed from the cage and only pairs that were mating were kept. The mating cage was devoid of any plant material and sugar water. During mating, the male: female ratio was 1:1 or 2:1 depending on the availability of adult moths. Only mated female moths were used for the oviposition experiments.

Plant Growth: The plant samples were grown in the greenhouse of the Max-Planck Institute. Wild type *Datura* and *Nicotiana* seeds were collected from the desert in Utah and then over generations were grown in greenhouse culture under 16 h of light, with temperature during day time set at 23–25° Celsius and at night 19–23° Celsius, humidity during the entire day was set to 50–60 percent. The seeds were initially potted in a germination tray. After 20 days the saplings were transferred to 1 L pots. Once the plants reached a height of roughly 50 cm (*Nicotiana*: 80 cm) they were used for the experiments. At least 1 week before the experiment plants were transferred to a climate chamber with the same settings as the moth flight cage.

Behavioral wind tunnel assay: In a wind tunnel (plexiglass, 250 cm × 94 cm × 90 cm; airflow, 0.4 m/s; light,

photosynthetic active radiation: 0.27 μmol/[m²s]; temperature, 25 °C; humidity, 70%) female hawkmoths were tested for their oviposition preference for *Datura wrightii* and *Nicotiana attenuata*. For every trial, a freshly excised leaf and a fresh moth were used. We selected leaves to match the surface area of the *Datura* and *Nicotiana* leaves in the choice assay.

Single leaves of both plants were placed on pillars (height of the pillar, 40 cm; diameter of each pole, 2 cm; distance between pillars, 45 cm) at the upwind end of the wind tunnel. Individual moths were kept in circular cages (diameter, 13 cm; height, 15 cm) in a pre-exposure chamber at the same temperature, humidity, and light as in the wind tunnel for about 1 h before the experiment.

For testing, the opened cages were placed on a take-off platform positioned at the downwind side within the wind tunnel. Each moth was given 2 min to initiate wing fanning (which usually precedes the moths' take-off) to be considered for analysis. After taking off, moths were recorded with five Logitech cameras (spatial resolution, 1280 × 720 pixel; temporal resolution, 30 frames per second).

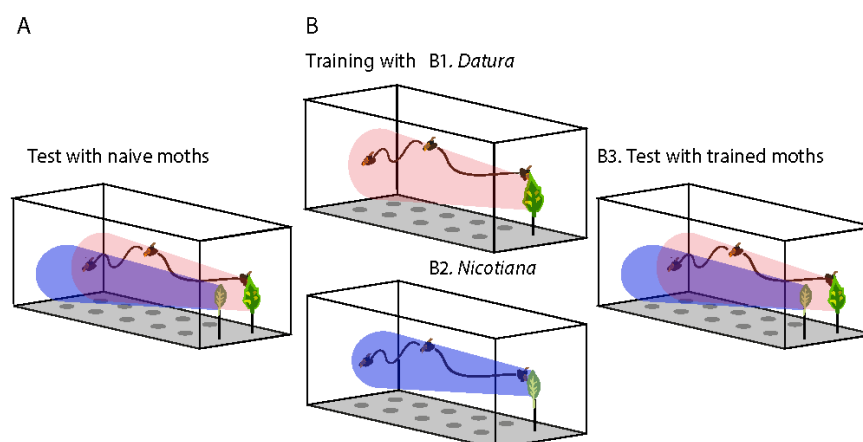
We asked the following four questions:

1. Do naïve ovipositing hawkmoths exhibit an innate preference for one of the host plants *Nicotiana attenuata* or *Datura wrightii* over the other?
Naïve moths were exposed in the wind tunnel to two individual leaves of *Datura wrightii* and *Nicotiana attenuata*.
During five minutes of flight, the first choice and the total number of eggs laid on each leaf were recorded (Fig. 1A)
2. Would oviposition experience alter a moth's oviposition preference?
and, if so,
3. How long would the memory last?

Naïve moths were exposed to a single leaf of either *Datura wrightii* or *Nicotiana attenuata* (Figs. 1B1 and 2) in the wind tunnel. As soon as the oviposition started, the animals were captured, resulting usually in a single egg (at max six eggs) laid before the moth was removed from the wind tunnel. These from now on called "experienced moths" were tested for their oviposition preference either 30 min after their oviposition experience, or 1 day later: As before, the now experienced moths were exposed in the wind tunnel to two individual leaves of *Datura wrightii* and *Nicotiana attenuata*. Again, during five minutes of flight, the first choice and the total number of eggs laid on each leaf were recorded (Fig. 1B3).

4. Does experience increase oviposition efficiency?

Fig. 1 Experimental paradigm to test for any effect of experience on the moths' oviposition preference. **A** Innate preference; Test for the moths' innate oviposition preference for single leaves of *Datura* and *Nicotiana*. **B** Learned preference; Moths are allowed to oviposit once on either *Datura* (**B1**) or *Nicotiana* (**B2**) and their preference for single leaves of *Datura* and *Nicotiana* becomes tested afterward (**B3**)



As mentioned above, naïve moths were exposed to leaves of either *Datura* or *Nicotiana* in the wind tunnel, and the time from starting flight until they laid their first egg was measured. The same – now experienced – moths were 30 min later again exposed to a leaf of the trained plant and the time from starting flight until laying the first egg was measured (experimental set up as in Fig. 1B).

All data obtained were first checked for normal distribution. Since data were not normally distributed we performed non-parametric tests for statistical analysis. Details of statistics are provided in figure legends.

Results

Moths exhibit an innate preference for *Datura*: To test whether gravid females exhibit an innate preference, we offered naïve females single leaves of *Datura* and *Nicotiana* in a 2-choice assay in a wind tunnel. While the moths contacted both leaves equally often during the first leaf investigation (Naïve; Fig. 2), the females laid more eggs on *Datura* than on *Nicotiana* leaves during the 5 min of observation (Naïve; Fig. 2). We conclude that visual and olfactory cues of *Datura* and *Nicotiana* are equally strong to attract naïve females over distance, but that, when it comes to oviposition, *Datura* leaves are more attractive to the moths than those of *Nicotiana*.

Learning alters oviposition preference: When we tested experienced moths, i.e. moths that had already oviposited either on a *Datura* or *Nicotiana* leaf, we found altered preferences when compared to naïve moths. Moths that were tested 30 min after they had experienced a leaf of a given plant species, exhibited a strong preference for this species regarding their first choice (Mid-term memory; Fig. 2). In addition, moths that experienced *Datura*, exhibited an even

increased oviposition preference for *Datura* as compared to naïve moths. Moths that, however, experienced the innately less preferred *Nicotiana* later reversed their oviposition preference towards this plant (Mid-term memory; Fig. 2). While the increased preference for *Datura* after experiencing it once remained even after 24 h, the learned preference for *Nicotiana* disappeared after this time period (Long-term memory; Fig. 2).

In order to test, whether the experience does not only change the moths' preferences but also increases their oviposition efficiency, we tested, whether experienced moths would approach a given leaf and oviposit on it faster than naïve moths. While experience with *Nicotiana* did not result in any increased oviposition efficiency, moths that had oviposited on a *Datura* leaf once, later took significantly less time to approach the leaf and oviposit on it (Fig. 3).

Discussion

Our findings show that learning alters the oviposition preference in mated female *Manduca sexta* moths. Naïve moths, when exposed to individual leaves of *Datura wrightii* and *Nicotiana attenuata*, do not exhibit any preference regarding their first choice but finally lay more eggs on the *Datura* leaf. The latter is in agreement with another study, where hawkmoths, when tested with whole plants of four species including *Datura* and *Nicotiana*, deposited most of their eggs on the *Datura* plants (Späthe et al. 2013b). This oviposition preference might be due to visual and/or olfactory cues. However, it is of course also possible that the moth's final decision to lay an egg is driven by tactile, i.e. gustatory or physical, cues after the moth has contacted the leaf. In field experiments, *Manduca sexta* females exhibited an equally strong preference to oviposit on *Datura* and *Nicotiana* plants as compared to other potential host plants

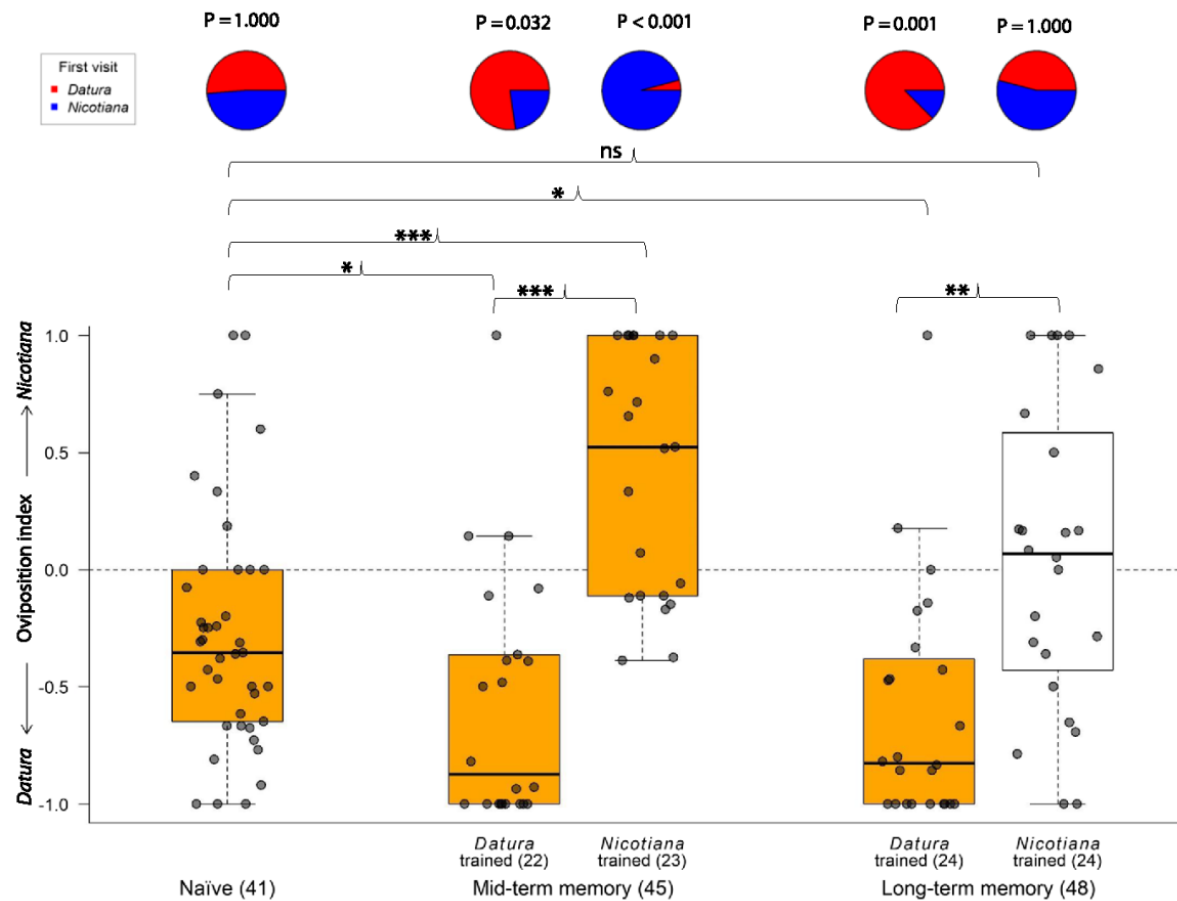


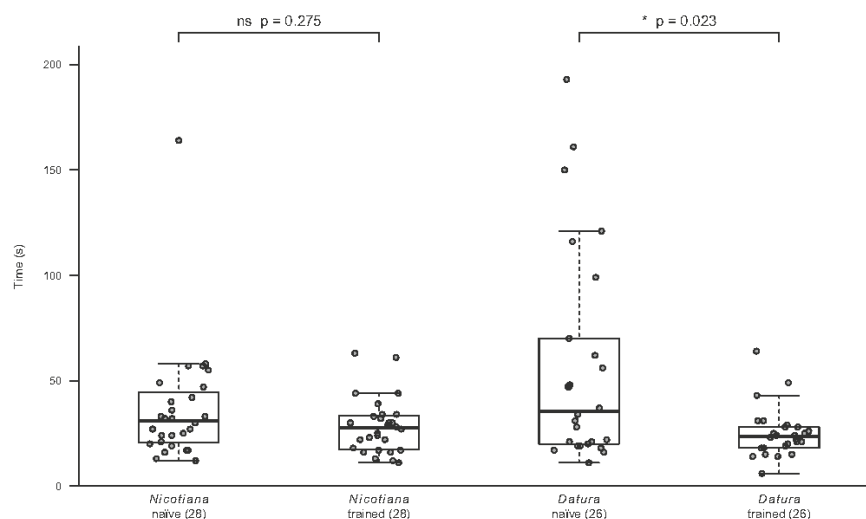
Fig. 2 *Manduca sexta*'s ovipositing preference can be altered by experience. First choices (pie charts) and oviposition indices (box-plot diagrams) of naïve and experienced *Manduca* females. Left panel, naïve moths; middle panel, experienced moths tested 30 min after experiencing either *Datura* or *Nicotiana*; right panel, experienced moths tested 1 day after experiencing either *Datura* or *Nicotiana*; sample sizes for each group are given in brackets. Oviposition index for each moth was calculated as (number of eggs on

Nicotiana—number of eggs on *Datura*)/total number of eggs. First choice: Pie charts, p-values below 0.05 depict significant preference during the first choice. Oviposition index: colored box plots depict an index different from 0 ($p < 0.05$, Wilcoxon signed rank test). Boxplots give the median (black line), quartiles (box), and 95% confidence intervals (whiskers). Brackets between boxplots depict differences between treatment groups (Kruskal Wallis analysis with selected pairwise Wilcoxon rank sum test corrected for repeated analyses)

(Garvey et al. 2020). It is usually assumed that female moths prefer host plants on which their offspring performs best. Larval performance depends both on the nutritional value of the plant and on potential protection the larvae get from the plant against predators and parasitoids. *Manduca sexta* was e.g. shown to prefer *Nicotiana* over *Solanum* plants, although their larvae grow much faster on *Solanum*. The reason for that might be the significantly lower parasitization rate of *Manduca* larvae when they grew on *Nicotiana* plants (Garvey et al. 2020). The reason for the innate preference for *Datura* described in our study remains open. In our lab colony, we provide females always with *Datura* plants for oviposition. By that, we might have selected for a *Datura* preference in the breeding situation.

However, after having a single chance to oviposit on a given leaf of only one of the two plants, and being then offered leaves of both species again, females immediately choose the leaf of the plant they have experienced already, and during 5 min of flight deposit a significantly higher number of eggs on this leaf (Fig. 2). It seems that the oviposition experience drives the moth's preference during consecutive oviposition events. We also show, that a single oviposition experience is enough to affect the moth's preference even after 24 h (Long-term memory; Fig. 2). Interestingly, this holds true for the experience with *Datura* only, where the first choice of the moths was still significantly turned towards this plant 24 h after ovipositing on it. Obviously, the learning of the innately less preferred *Nicotiana*

Fig. 3 Experience can increase oviposition efficiency. Time from starting flight until oviposition in naïve and experienced moths. Boxplots give the median (black line), quartiles (box), and 95% confidence intervals (whiskers). Square brackets, p-values below 0.05 depict significant differences between naïve and experienced moths (Wilcoxon matched-pairs rank sum test). The sample size for each group is given in brackets



plant is weaker, as the strong preference that we observed 30 min after the experience with *Nicotiana* was completely abolished after 24 h. It remains open, whether the moths' memory for *Datura* lasts longer, because of any potential selection for *Datura* preference during the breeding process (*Datura* is provided for oviposition in our breeding colony). Future studies shall reveal, whether maybe even just the exposure to a host plant odor without any oviposition experiences can affect the moths' later preference and whether repeated oviposition on a given plant species even increases the memory duration.

To our knowledge, a comparatively fast learning of oviposition cues so far has been shown only in parasitoid wasps (Kaiser et al. 2003). When the wasp *Leptopilina boulardi* oviposits in a *Drosophila* larva while being exposed to a specific odorant, it associates this odorant with the host, and will later target this olfactory cue again. While *L. boulardi* needs several training trials to form a long-term memory, the close relative *L. glomerata*, requires only one trial to still remember the odorant after one day (Smid et al. 2007). With keeping their increased preference for *Datura* despite a single oviposition experience even after 24 h, *Manduca sexta*' learning capabilities seem to match those of *L. glomerata*.

The formation of an associative memory between a given odor and food has been investigated in many other insects including (among others) ants (Huber and Knaden 2018), wasps (El-Sayed et al. 2018), and even mosquitos (Tomberlin et al. 2006). Also, *M. sexta* has been shown to increase its preference for the odor of innately less attractive flowers after feeding on them (Cook et al. 2020; Riffell et al. 2008). Learning of food odors can thus result in food constancy, where the insect focuses its foraging behavior on food sources it had good experience with.

Our knowledge regarding such constancy behavior in herbivorous insects is mainly limited to flower constancy. Here, insect pollinators tend to restrict their visits to those flower types they have already successfully fed from, and at the same time ignore potentially rewarding flowers they have no experience of. Different explanations of why flower constancy could be adaptive for the insect have been well reviewed (Amaya-Márquez 2009). One possible explanation is that insects can handle only limited information at a time, such as suggested by the 'short memory limitation theory'. Another possible explanation comes from the 'learning investment hypothesis' (Chittka et al. 1997, 1999; Menzel et al. 1998; Raine and Chittka 2005), which predicts that the investment required by an insect to learn how to handle a new flower type is higher than the possible gain coming from a switch in flower type. Overall, switching between flowers with different morphologies can increase total flower handling time. Therefore, foraging insects might remain flower-constant to optimize their costs per benefit (Chittka et al. 1997; Lavery 1994).

While learned flower constancy is well investigated, less is known about learned oviposition constancy as we describe in the present study. Finding a suitable host plant for oviposition is crucial for the fitness of herbivorous insects as it affects the survival of their offspring. It was shown before that the generalist *Spodoptera littoralis* preferentially oviposits on host plants it fed on during its larval stage (Lhomme et al. 2020). Obviously, upon feeding on a given host plant, the larva learns host-specific cues, which drive its oviposition behavior when it becomes an adult. In this case, larval feeding can be interpreted as the rewarding act that leads to a shift in host-plant preference. It was also shown, that moths that as adults experienced a host plant for several days, later preferred this species over other host plant species

they had not experienced before (Cunningham et al. 1998). In our case, however, the adult hawkmoth changes its preference after a single oviposition event. To oviposit on a given host plant, the moth first follows the plume of the plant, targets the leaf by both vision and olfaction, then probes it with the legs and the ovipositor which might provide both gustatory as well as mechanosensory information, before it finally decides whether or not to lay an egg. We show that the decision to lay an egg results in a strong preference to target the same host plant species again. As we performed all experiments with real leaves, we cannot tell, whether the learned preference is mainly driven by visual or olfactory cues. Further investigations will also reveal which part of the oviposition process is rewarding to the moth and finally leads to the shift in preference. Is it the process of the actual egg delivery or is any kind of sensory feedback the moth experiences upon first leaf investigation sufficient to later make the moth target the same plant again? And what are the cues that dictate, whether the moth still remembers one plant after 24 h (*Datura*), but forgets about the other (*Nicotiana*)?

Despite these open questions, we can still answer, whether the moth potentially profits from its learned oviposition constancy. When experiencing a leaf of a given plant once, the female moth probably does not learn too much about, whether this plant is good or not for the performance of the moth's offspring. This knowledge should be rather inherited and should govern the innate oviposition preference of the female moth. Any experience the moth gets during oviposition should rather affect the female's own future performance. In the wind tunnel naïve moths took more time to target a *Datura* leaf as compared to a *Nicotiana* leaf. This might be due to the comparatively low amount of volatiles released by *Datura* (Späthe et al. 2013a). However, animals that had experienced *Datura* already, targeted the olfactory inconspicuous *Datura* leaves significantly faster than naïve animals (Fig. 3). It could be that experience led to the formation of a search image – as also discussed for bees when establishing flower constancy (Amaya-Márquez 2009). Since flight for a gravid female is costly, both in terms of energy as well as due to potential predators, learning that increases the efficiency in targeting host plants could be seen as adaptive. An increasing oviposition efficiency due to experience has also been observed in three species of *Colias* butterflies. Females of these species lay single eggs on legume plants and when searching for further hosts become more and more efficient in discriminating legume plants from visually similar non-host plants (Stanton 1984). A similarly increased efficiency to locate a host plant after oviposition experience with this plant was also shown in two other generalist butterflies (*Polygonia c-album* and *Vanessa cardui*). Both became faster in localizing a given host among non-host plants after they had gained oviposition experience on it (Gamberale-Stille et al. 2019). One should mention, that

the female moths in our experiments experienced isolated leaves only. This is of course an artificial situation, as in the field both *Datura* and *Nicotiana* are visited by the moths for oviposition and nectar feeding. It is furthermore known that nectar amount and nectar chemistry can affect the moths subsequent oviposition behavior on that plant (Adler and Bronstein 2004). While our findings suggest that oviposition experience is sufficient to change the moths' oviposition preferences, feeding experience at the flowers of those plants may have further effects.

Finally, to survive, insects should be able to learn and adapt to changes in the ecosystem. With varying climatic conditions, host plant species diversity tends to change. Fast flying moths like *Manduca sexta* might during their life therefore experience habitats with different host plant species. A non-changeable preference for a given host plant could hence result in reduced numbers of offspring if a female moth ends up in a habitat that does not provide this specific plant. At the same time, human-derived pollutions like ozone and nitric oxides have been shown to change plant emissions (Farré-Armengol et al. 2016; Fuentes et al. 2016). It was shown that *Manduca sexta* can learn ozone-induced changes in floral odors (Cook et al. 2020), and might therefore still be able to forage despite the degrading effects pollutants have on its innately attractive flower odors. We do not know yet, whether olfactory driven host choice is affected by degrading pollutants also. A flexible host preference, however, that can be changed upon experience might help insects to locate suitable hosts even under these less predictable conditions.

Author's Contribution Study design: NN, BSH, and MK designed the study; NN and EA conducted experiments; NN, EA, and MK, analyzed data. NN wrote first draft of the manuscript; All authors edited the manuscript.

Funding The study was funded by the Max Planck Society. Open Access funding enabled and organized by Projekt DEAL.

Data Availability All data are presented in the manuscript.

Code Availability (Software Application or Custom Code) Not applicable.

Declarations

Conflicts of Interest/Competing Interests The authors declare no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated

otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Adler LS, Bronstein JL (2004) Attracting antagonists: does floral nectar increase leaf herbivory? *Ecology* 85:1519–1526
- Amaya-Márquez M (2009) Floral constancy in bees: a revision of theories and a comparison with other pollinators. *Revista Colombiana De Entomología* 35:206–216
- Bisch-Knaden S, Dahake A, Sachse S, Knaden M, Hansson BS (2018) Spatial representation of feeding and oviposition odors in the brain of a hawkmoth. *Cell Reports* 22:2482–2492
- Chittka L, Gumbert A, Kunze J (1997) Foraging dynamics of bumble bees: correlates of movements within and between plant species. *Behav Ecol* 8:239–249
- Chittka L, Thomson JD, Waser NM (1999) Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften* 86:361–377. <https://doi.org/10.1007/s001140050636>
- Cook B, Haverkamp A, Hansson BS, Ta Roulston, Lerdau M, Knaden M (2020) Pollination in the Anthropocene: a moth can learn ozone-altered floral blends. *J Chem Ecol* 46:987–996
- Cunningham JP, Jallow MF, Wright DJ, Zalucki MP (1998) Learning in host selection in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Animal Behaviour* 55:227–234
- Delaney KJ, Macedo TB, Peterson R, Hingley L (2001) The impact of herbivory on plants: yield, fitness, and population dynamics Biotic stress and yield loss:135–160
- El-Sayed AM, Jösvai JK, Brown RL, Twidle A, Suckling DM (2018) Associative learning of food odor by social wasps in a natural ecosystem. *J Chem Ecol* 44:915–921
- Farré-Armengol G, Peñuelas J, Li T, Yli-Pirilä P, Filella I, Llusia J, Blande JD (2016) Ozone degrades floral scent and reduces pollinator attraction to flowers. *New Phytol* 209:152–160
- Fuentes JD, Chamecki M, Ta Roulston, Chen B, Pratt KR (2016) Air pollutants degrade floral scents and increase insect foraging times. *Atmos Environ* 141:361–374
- Gamberale-Stille G, Schäpers A, Janz N, Nylin S (2019) Selective attention by priming in host search behavior of 2 generalist butterflies. *Behav Ecol* 30:142–149
- Garvey MA, Creighton JC, Kaplan I (2020) Tritrophic interactions reinforce a negative preference–performance relationship in the tobacco hornworm (*Manduca sexta*). *Ecol Entomol* 45:783–794
- Goulson D, Stout JC, Hawson SA (1997) Can flower constancy in nectaring butterflies be explained by Darwin's interference hypothesis? *Oecologia* 112:225–231
- Gruter C, Ratnieks FL (2011) Flower constancy in insect pollinators: Adaptive foraging behaviour or cognitive limitation? *Commun Integr Biol* 4:633–636. <https://doi.org/10.4161/cib.16972>
- Haverkamp A, Bing J, Badeke E, Hansson BS, Knaden M (2016). Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. *Nature Comm* 7:11644.
- Huber R, Knaden M (2018) Desert ants possess distinct memories for food and nest odors. *Proc Natl Acad Sci* 115:10470–10474
- Kaiser L, Perez-Maluf R, Sandoz J, Pham-Delègue M-H (2003) Dynamics of odour learning in *Leptopilina boulardi*, a hymenopterous parasitoid. *Anim Behav* 66:1077–1084
- Knauer AC, Schiestl FP (2015) Bees use honest floral signals as indicators of reward when visiting flowers. *Ecol Lett* 18:135–143
- Laverty TM (1994) Bumble bee learning and flower morphology. *Anim Behav* 47:531–545
- Lhomme P, Khallaf M, Larsson M, Anderson P (2020) A sensitive period for the induction of host plant preference in a generalist herbivorous insect. *Anim Behav* 169:1–8
- Menzel R, Geiger K, Joerges J, Müller U, Chittka L (1998) Bees travel novel homeward routes by integrating separately acquired vector memories. *Anim Behav* 55:139–152
- Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? *Oikos* 120:321–326. <https://doi.org/10.1111/j.1600-0706.2010.18644.x>
- Raine NE, Chittka L (2005) Comparison of flower constancy and foraging performance in three bumblebee species (Hymenoptera: Apidae: Bombus). *Entomologia Generalis* 28:081
- Riffell JA, Alarcón R, Abrell L, Davidowitz G, Bronstein JL, Hildebrand JG (2008) Behavioral consequences of innate preferences and olfactory learning in hawkmoth–flower interactions. *Proceedings of the National Academy of Sciences* 105:3404–3409
- Smid HM, Wang G, Bukovinsky T, Steidle JL, Bleeker MA, van Loon JJ, Vet LE (2007) Species-specific acquisition and consolidation of long-term memory in parasitic wasps. *Proceedings of the Royal Society b: Biological Sciences* 274:1539–1546
- Späthe A, Reinecke A, Haverkamp A, Hansson BS, Knaden M (2013) Host plant odors represent immiscible information entities—blend composition and concentration matter in hawkmoths. *PLoS One* 8:e77135
- Späthe A, Reinecke A, Olsson SB, Kesavan S, Knaden M, Hansson BS (2013b) Plant species- and status-specific odorant blends guide oviposition choice in the moth *Manduca sexta*. *Chem Senses* 38:147–159
- Stanton ML (1984) Short-term learning and the searching accuracy of egg-laying butterflies. *Anim Behav* 32:33–40
- Tomberlin JK, Rains GC, Allan SA, Sanford MR, Lewis WJ (2006) Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae). *Naturwissenschaften* 93:551–556
- Waser NM (1986) Flower constancy: definition, cause, and measurement. *The American Naturalist* 127:593–603
- Zhang J et al (2019) The olfactory coreceptor IR8a governs larval feces-mediated competition avoidance in a hawkmoth. *Proceedings of the National Academy of Sciences* 116:21828–21833

Manuscript III: Fast Learners: One Trial Olfactory Learning in Insects

Elisabeth Adam, Bill S. Hansson, Markus Knaden[‡]

[‡]Author for correspondence (mknaden@ice.mpg.de)

Frontiers in Ecology and Evolution

Published: April 11th, 2022

Front. Ecol. Evol., 10:876596; doi: 10.3389/fevo.2022.876596

Since there has been some controversy in the field of insect learning as to how fast long term memories (LTMs) are formed, I decided to write a comprehensive review about this topic. In the past it has been believed that LTMs are only formed after repeated interspaced experiences of a neutral stimulus (e.g. an odor; conditioned stimulus, CS) together with a reward or punishment (unconditioned stimulus, US). Recent studies have, however, shown that insects are able to form LTMs after as little as one trial. In this review I discuss insect olfactory learning, long established odor conditioning protocols and recent studies that show that insects are incredibly fast learners. I further suggest to look at insect learning in an ecological context and draw the attention to factors that can influence the outcome of studies on insect learning.

Author contributions:

Written based on an idea conceived by all authors.

Wrote manuscript: EA (90 %), M.K., B.S.H.

FORMULAR 1

Manuskript Nr. III

Titel des Manuskriptes: Fast Learners: One Trial Olfactory Learning in Insects

Autoren: Elisabeth Adam, Bill S. Hansson, Markus Knaden

Bibliographische Informationen:

Adam, E., Hansson, B. S., & Knaden, M. (2022). Fast learners: One trial olfactory learning in insects. Review. Frontiers in Ecology and Evolution, 10:876596. <https://doi.org/10.3389/fevo.2022.876596>

Der Kandidat / Die Kandidatin ist (bitte ankreuzen)

☒ Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: publiziert

Anteile (in %) der Autoren / der Autorinnen an den vorgegebenen Kategorien der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Adam, E.	70 %	-	-	90 %	-
Hansson, B.S.	15 %	-	-	5 %	100 %
Knaden, M.	15 %	-	-	5 %	-
Summe:	100 %	-	-	100 %	100 %

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in (Mitglied der Fakultät)



Fast Learners: One Trial Olfactory Learning in Insects

Elisabeth Adam[†], Bill S. Hansson[†] and Markus Knaden^{*†}

Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Jena, Germany

OPEN ACCESS

Edited by:

Stefan Dötterl,
University of Salzburg, Austria

Reviewed by:

Sufang Zhang,
Research Institute of Forest Ecology,
Environment and Protection, Chinese
Academy of Forestry, China
Juergen Gross,
Julius Kühn-Institut, Germany

*Correspondence:

Markus Knaden
mknaden@ice.mpg.de

†ORCID:

Elisabeth Adam
orcid.org/0000-0003-4382-8994
Bill S. Hansson
orcid.org/0000-0002-4811-1223
Markus Knaden
orcid.org/0000-0002-6710-1071

Specialty section:

This article was submitted to
Chemical Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 15 February 2022

Accepted: 21 March 2022

Published: 11 April 2022

Citation:

Adam E, Hansson BS and Knaden M
(2022) Fast Learners: One Trial
Olfactory Learning in Insects.
Front. Ecol. Evol. 10:876596.
doi: 10.3389/fevo.2022.876596

Despite their comparatively small brains, insects are able to survive and thrive in their environment. In the past, it was thought that insects are driven mainly by their instincts. However, today it is well established that they possess unique abilities to learn and use their experience in future decisions. Like many higher animals insects are able to acquire and retain information on when and where to forage, which mate to choose, where to lay their eggs and how to navigate in complex habitats. Learning can be surprisingly fast with only one single encounter with a suitable food source or oviposition site shaping an insect's preference for up to a lifetime. In this review, we discuss the scope and limits of insect learning, focusing in specific on olfactory learning, and we raise the question whether currently used learning paradigms in artificial lab set-ups are able to answer all ecologically relevant questions.

Keywords: insect learning, one trial learning, ecology, insect cognition, insect memory, olfaction, olfactory learning

INTRODUCTION

"This tiny insect has a brain?!" There is hardly any researcher working on insect behavior, neurobiology or cognition that hasn't heard this sentence. We patiently answer, that yes indeed, insects have a central nervous system and are able to learn, remember past experiences, and navigate in complex environments that require the integration of different sensory modalities (In less scientific jargon for the non-scientific community). Research of the past years has shown that insects not only possess short-, mid- and long-term memory, but also are capable of learning concepts, innovating, transmitting preferences from one generation to the next or learning by observing others (Leadbeater and Chittka, 2007; Avarguès-Weber and Giurfa, 2013; Giurfa, 2013; Chittka et al., 2019; Lihoreau et al., 2019). These abilities are important in all areas of life of the insect such as foraging, social interactions, reproductive behavior, and predator avoidance (Dukas, 2008; Dion et al., 2019). Since several reviews already cover the history and recent developments of insect cognition and learning, this will not be part of our review. For more information on these topics see: Dukas (2008), Giurfa (2013), Giurfa (2015), and Lihoreau et al. (2019). Here, we focus on olfactory-driven learning and how insects are able to use their unique cognitive abilities to learn essential information (for survival) in often as little as a single trial.

INSECT COGNITION

When insects interact with their environment, they constantly acquire and store new information. However, only if the information is retained over a longer period of time and results in a corresponding change of the insect's behavior, this is regarded as learning and memory (Papaj and Prokopy, 1989). Neither the size of the insect's brain nor the relatively short life span should, however, be considered constraints when it comes to learning. Studies in the past have shown that

learning is possible even with a small neuronal network (Van Lenteren and Bakker, 1975; Laverty, 1980; Hammer and Menzel, 1995; Keene and Waddell, 2007) and the process of learning itself might not take more than a few seconds or minutes, which will still give the animal ample opportunity to utilize the newly acquired knowledge during its (short) lifetime (Dukas, 2008). When thinking of insect learning, fruit flies (*Drosophila melanogaster*) and honeybees (*Apis mellifera*) are small, but well-established insect model organisms for learning and memory (Davis, 2005; Giurfa and Sandoz, 2012; Menzel, 2012). Still, there are insects with even more minuscule brains that are able to learn: Parasitoid wasps are among the smallest insects on earth, with brains measuring only a few cubic micrometers but are, nevertheless, able to associate odors with a reward after as little as one trial (Van Der Woude et al., 2018). In the wild, these tiny insects use their ability to learn odors to recognize the olfactory bouquet of damaged plants that indicate their herbivorous hosts (Haverkamp and Smid, 2020).

OLFACTORY LEARNING AND CIRCUIT

In contrast to humans, many insects heavily rely on olfactory rather than visual information to learn new things about their environment. The main advantage is that odors can be perceived over greater distances than visual cues and that they can be traced back to the source even when the view of the source is obstructed. Therefore, insects often utilize them to navigate toward foraging, oviposition or nest sites. Desert ants (*Cataglyphis fortis*), for example, navigate successfully in the arid environment of the Tunisian salt pans. Although they are known to use path integration and visual cues to find their way in the vast landscape, they also rely on olfactory landmarks to locate their nest or promising food sources (Knaden and Graham, 2016). Odors do also play a role in social interactions of insects: Honeybees communicate with conspecifics through pheromones, use olfaction to locate flowers and communicate olfactory information about possible food sources with other bees in the hive (Paoli and Galizia, 2021). Blowing learned odors into the bee hive is enough to trigger the bees to fly out and search for food at foraging sites that have previously been associated with this specific odor (Reinhard et al., 2004). Apart from using olfaction to navigate the environment and localize food sources, odors can also be learned and remembered in a mating context. Male fruit flies (*D. melanogaster*) are able to learn which mates to choose and to connect odor information to the receptivity of the other sex. Males transfer their pheromone *cis*-vaccenyl acetate (cVA) to females during mating. Other male flies innately show reduced courtship toward these freshly mated females as those now emit the male pheromone cVA. However, if the male additionally experiences rejection by a mated female emitting cVA, male courtship behavior is subsequently further reduced, suggesting that the innate courtship inhibition of cVA can be further enhanced by learning (Keleman et al., 2012).

Olfactory memory can be categorized in two main categories: Appetitive memory and aversive memory (Figure 1). Appetitive memory is formed if a conditioned stimulus (CS), i.e., the odor,

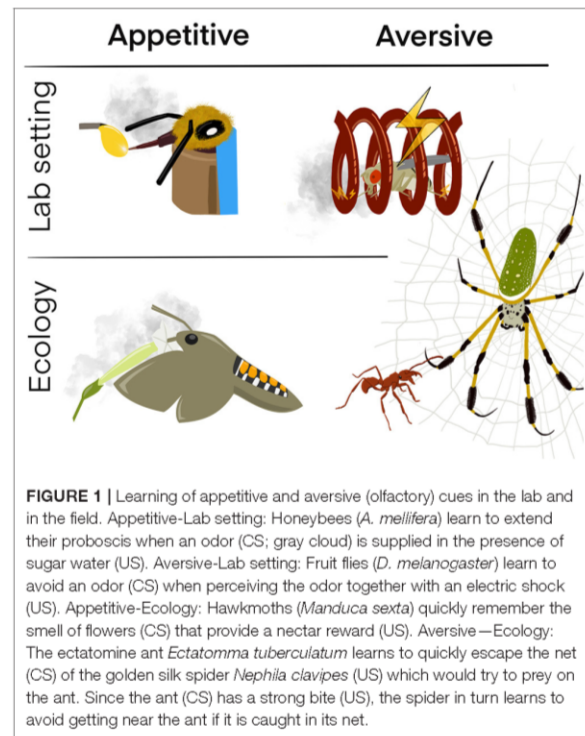
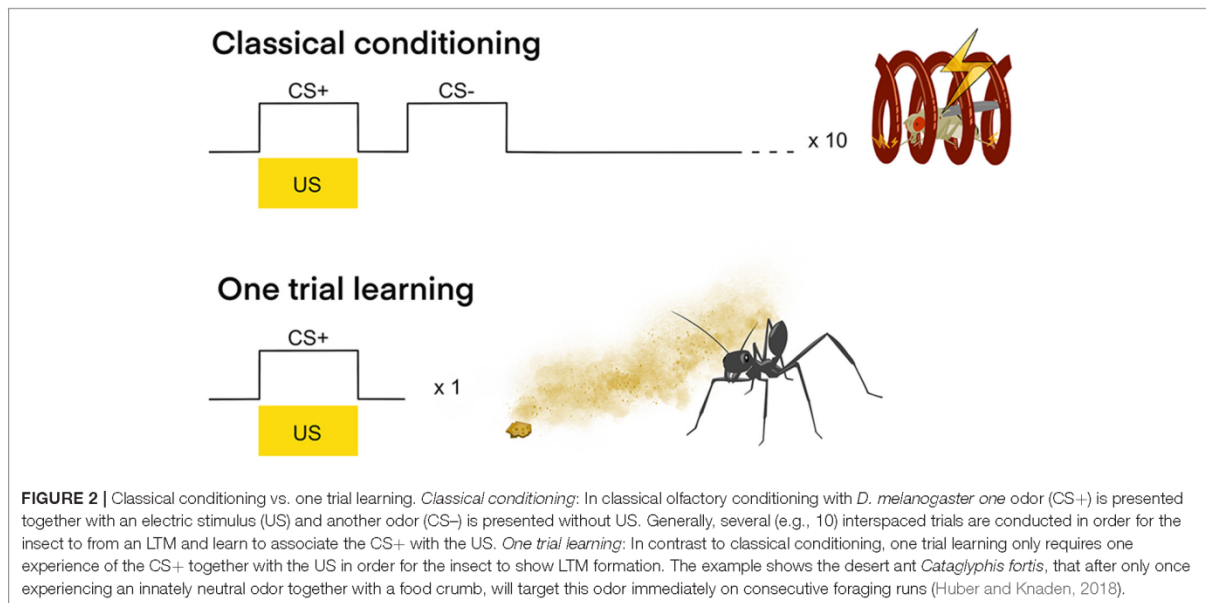


FIGURE 1 | Learning of appetitive and aversive (olfactory) cues in the lab and in the field. Appetitive-Lab setting: Honeybees (*A. mellifera*) learn to extend their proboscis when an odor (CS; gray cloud) is supplied in the presence of sugar water (US). Aversive-Lab setting: Fruit flies (*D. melanogaster*) learn to avoid an odor (CS) when perceiving the odor together with an electric shock (US). Appetitive-Ecology: Hawkmoths (*Manduca sexta*) quickly remember the smell of flowers (CS) that provide a nectar reward (US). Aversive—Ecology: The ectatomine ant *Ectatomma tuberculatum* learns to quickly escape the net (CS) of the golden silk spider *Nephila clavipes* (US) which would try to prey on the ant. Since the ant (CS) has a strong bite (US), the spider in turn learns to avoid getting near the ant if it is caught in its net.

is associated with a positive unconditioned stimulus (US) like a sugar reward. Aversive memory is formed if the CS is associated with a negative US such as an electric shock or a bitter compound (Kahsai and Zars, 2011). Both types of memory are key for the survival of a species as it is important to approach possible food sources (appetitive) and avoid dangerous substances such as toxins (aversive). To better understand where such associations are formed, let us briefly introduce the detection and early processing of olfactory information in the insect brain. When smelling odors, insects detect the volatile compounds with fine structures, so-called “sensilla”, on their antennae and maxillary palps. These sensilla house sensory neurons expressing odorant receptors that determine to which odorant(s) a given neuron will respond to. When an odorant binds to a fitting receptor, the neuron becomes activated and the signal is sent from the antennae or maxillary palps to the central brain of the insect, where it is being processed. The first processing center of the brain is the antennal lobe (AL). From there the odor information is passed on by projection neurons (PNs) to higher brain regions like the mushroom body (MB) or the lateral horn (LH). While the latter is known to mainly drive innate olfactory behavior, the MB acts as a coincidence detector that helps the insect to learn to associate a given odor with a reward or punishment (Carey and Carlson, 2011). The mushroom body output neurons (MBONs) which are connected to the MB control the approach or avoidance of an olfactory cue, with dopaminergic neurons (DANs) encoding the positive or negative valence of a stimulus



(Cognigni et al., 2018). Recent work from Eschbach et al. (2021) in *D. melanogaster* has further shown that there are connections between the neurons of the LH and MBONs and that downstream of the MBONs a distinct subset of convergence neurons (CNs) is integrating innate and learned valences. Depending on which valence is higher, the insect will show different behaviors. That means that even though a naïve insect might perceive an odor as innately attractive, there is the possibility to alter this innate preference by increasing the negative valence of the odor through learning (e.g., through punishment while smelling the odor). This in turn will alter the insect's behavior from approach to avoidance.

MEMORY FORMATION—A CLASSICAL VIEW

When it comes to storing information, four different types of memory have been described: Short-term memory (STM), mid-term memory (MTM), long-term memory (LTM), and anesthesia-resistant memory (ARM) (Dezazzo and Tully, 1995). The difference between LTM and ARM is that LTM formation is associated with new protein synthesis after training and can be disrupted by amnesia-inducing agents while ARM is/cannot (Tully et al., 1994). Recent studies on *D. melanogaster* have shown that the transcription factor Apterous (Ap) is needed for memory consolidation, that is the formation of a stable memory (Inami et al., 2021), and that memory consolidation requires neuronal activity beyond the learning experience (Cognigni et al., 2018). Inami et al. (2021) have further shown that both Ap and its cofactor Chip (Chi) are required for the maintenance of LTMs in the MB. When looking at the neuronal circuits involved, acquisition and expression of very early memories take place in

the so-called γ neurons of the MB. For ARM and LTM formation the vertical lobes of the MB, α' and β' or α and β , respectively, play the most important role (Güven-Ozkan and Davis, 2014). The exact coding of the memories seems, however, to be more complex than first suggested and might include several feedback loops, some of which still need to be elucidated (Cognigni et al., 2018).

In the past it was believed that in order for LTMs to form, the insect has to experience CS and US together multiple times and that these experiences also have to be spaced apart over time (Dezazzo and Tully, 1995). This view is, however, being challenged by a mounting body of evidence that insects are able to form LTMs after just a single trial.

MEMORY FORMATION—SIMILAR, YET DIFFERENT?

One of the most important model organisms to shed light on different types of memory and the corresponding expression of related genes is the fruit fly (*D. melanogaster*). It offers the necessary tool kit to connect genetic information with the activity of neuronal networks and behavior (Davis, 2005). Classical (Pavlovian) aversive olfactory conditioning is usually conducted with groups of flies. These flies are exposed to two different odors, one odor (CS+) is paired with an electric shock (US), and the other odor (CS-) is not. After exposure, the flies are tested in a T-maze where they have to choose between the two different odors (Tully and Quinn, 1985). In *D. melanogaster* one trial learning was thought to lead to the retention of memory for up to a few hours only (Keene and Waddell, 2007). Also, olfactory LTM was thought to require 10 spaced trials, in 15 min intervals, with the CS (odor) being presented at the same time as the US (electric

shock) (Figure 2) (Tully et al., 1994). Several recent studies on fruit fly (*D. melanogaster*) memory and behavior have drawn a quite different picture when it comes to the formation of LTM.

In their study, Krashes and Waddell (2008) could show that using a new appetitive conditioning protocol, LTM could be formed after a single trial. The authors exposed starved flies to an odor without sugar reward followed by another odor with sugar reinforcement. Flies were then further starved until testing (but had access to water). Olfactory memory for the reinforced odor persisted until 36 h. As most of the flies perished after that (presumably of starvation), longer memory retention could not be assessed, but seems very likely. Especially so, since protein synthesis inhibitor lowered memory retention, which shows that LTM was indeed formed. What is important to know about the authors' findings is that if flies were fed between training and testing, memory performance went down drastically. This indicates that motivation is a key aspect in memory retention since continuously starved flies performed very well during the memory test.

Motivation also seems to be the key aspect in water-reward memory in *D. melanogaster*. Water-reward LTM (wLTM) can be formed after a single trial and lasts more than 24 h. It is associated with *de novo* protein synthesis and CREB-mediated gene transcription in the α and β surface and γ dorsal neurons of the MB (Lee et al., 2020). Serotonergic neurons called dorsal paired medial (DPM) neurons modulate MBON neural activity in order to consolidate wLTM in the fly brain (Lee et al., 2021). However, only thirsty flies remember an odor (CS) presented together with a water reward (US). Since food-starved flies do not remember odors that are presented together with water, the motivation needed for memory formation also seems to be specific for the circumstance (Lin et al., 2014). *D. melanogaster* larvae are also able to form associations between an odor (CS) and a sugar reward (US) after a single trial. In contrast to the adult flies, they do, however, not form LTMs, but only STMs. It has no influence whether the US is a sugar reward that is sweet and nutritious (fructose), solely sweet (arabinose) or solely nutritious (sorbitol) (Weiglein et al., 2019). This raises the question how the ecology of the larvae is playing into their ability to store and retain information. Given that the larvae spend their whole life until pupation most likely in the same fruit, it might not be of importance for the larvae to associate a food odor with the fruit's sugar content. When looking at aversive cues, Weiglein et al. (2019) found that aversive STM is only formed for high-concentration sodium chloride but not for a bitter tastant (quinine). Again, here the ecology of the larvae could play a role. Adult *D. melanogaster* females are known to detect bitter compounds with their labellum and legs and lay their eggs in overripe fruit low in bitter compounds (Dweck et al., 2021). Hence, if the larvae never leave the fruit they hatched in, they do not need to assess the palatability of the fruit in terms of bitterness. Desiccation is, in contrast, a serious threat to a fly larva. Therefore, learning possible desiccation cues like an odor (CS) associated with high-concentration sodium chloride (US) might be essential for survival.

Also, in the second important insect model used for learning and memory, the honeybee, different types of memory have been

thoroughly investigated. As in *D. melanogaster*, LTM was thought to require a long consolidation phase and multiple spaced trials. Ecologically speaking, it was argued, this might assure that only very important memories get stored. For example when a bee encounters the same rewarding flower species multiple times during a foraging bout (Menzel, 1999). Still, one-trial learning has already been mentioned as a side note in a very early publication: Takeda (1961) observed that when using the classical appetitive olfactory conditioning approach, some honeybees showed the proboscis extension reflex (PER) after a single trial. A more recent study has shed light on this discrepancy in information.

In their study, Villar et al. (2020) could show that honeybees were underestimated when it comes to their ability to form LTMs. The authors found that a single appetitive training trial can induce LTM that persists several days and that protein-synthesis-dependent memories are formed as early as 4 h after the trial. So why do the authors see such a drastic difference to previous appetitive conditioning protocols? Honeybees are usually captured at the hive entrance, or worse, taken directly out of the beehive for testing. That means there is no control for whether the bee is a forager, builder, nurse or cleaner in the hive. Yet, only foragers are highly motivated to find and learn food sources. To control for selecting only true foragers, Villar et al. (2020) caught foragers at the feeder to ensure foraging motivation. The authors only performed experiments when the field conditions allowed for high motivation in foragers. That means they did not run experiments during bad weather, winter or periods of Asian-hornet predation. Further, a novel olfactometer was used that allowed a more precise temporal control of the stimulus presentation and thereby significantly reduced odor generalization.

The knowledge about memory formation in these model organisms is of incomparable value. Still, there seems to be more to the acquisition and retention of olfactory memories than can be shown with strict classical olfactory conditioning. Especially if one looks at the diversity of insect species and their different ecologies, the question arises if "one shoe fits all."

LEARNING RATES, ABILITIES AND PREDISPOSITIONS

Different insect species, even when phylogenetically closely related, can exhibit different learning rates and abilities (Smid et al., 2007). The closely related wasp species pair *Cotesia glomerata* and *Cotesia rubecula* show natural differences in foraging behavior. While *C. glomerata* is a gregarious generalist that lays up to 20 eggs in its host *Pieris brassicae*, the solitary *C. rubecula* is considered a specialist and only deposits one single egg into its host *Pieris rapae*. *Cotesia glomerata* usually encounters many of its hosts in a single plant, making the encounter highly predictable, while *C. rubecula* only will find one host per plant and has to search a variable environment for its host species. Of course, this means that the predictability of a host encounter is very different for both species. Obviously, this has led to very different learning abilities: While *C. glomerata* is able to form LTM after a single trial, *C. rubecula* needs three trials that

are temporally spaced apart to form LTM. The speed of memory consolidation also varies drastically between the two species. While *C. glomerata* only takes 4 h to consolidate LTM, *C. rubecula* takes up to 3 days (Smid et al., 2007). That does not mean that one species is a “bad” and the other one a “good learner.” It just means that different learning types have to be seen in an ecological context. If the environment is highly unpredictable, long-lasting memories might not be an advantage. In this case, short lasting memories that are updated continuously, or even an active form of forgetting, can be adaptive. The same goes in the other direction. If the environment is very stable and predictable, long lasting memories are more advantageous (Smid and Vet, 2016).

The ecology of the insect should also be considered when it comes to which type of cues are learned or in which context the cue is learned. The desert ant *Cataglyphis fortis* is known for its survival in the arid salt pans of North Africa where food is scarce and ants have to run far to find it. That means if the ant encounters a food item it is of high importance to learn how it smells quickly in order to be able to recognize possible similar food sources from afar (Figure 2). The ant also needs to be able to remember different types of food odors since it cannot always predict which type of food it is going to find on each consecutive foraging trips. There are times where e.g., cicadas or flying ant sexuals are quite abundant, but there are also times where hardly any dead insect found will be the same. Huber and Knaden (2018) could show that *C. fortis* is able to remember a food odor after only one experience, can remember at least 14 consecutively learned food odors, and can remember odors for up to a lifetime. Interestingly, the nest odor is learned completely differently by the species. It takes repeated learning trials to remember and is quickly forgotten if the odor is not present at the nest anymore (Huber and Knaden, 2018). This means that the same ant species learns the connection between an odor (CS) and a specific US in one trial during food search, but is incapable of this in another context, i.e., during nest search. What also needs to be thought of is that insects have different predispositions in terms of which cues they are able to learn easily and which cues a more difficult for them to learn. This type of learning has been described as “prepared learning” (Smid and Vet, 2016). In their study on the evolution of prepared learning Dunlap and Stephens (2014) could show that fruit flies (*D. melanogaster*) show increased sensitivity to (odor) cues that had been reliable over 40 generations than to cues that were not. This indicates that learning reliable cues can be adaptive for an insect and that these cues can become imperative to a species over generations.

ONE TRIAL LEARNING IN DIFFERENT SPECIES/ECOLOGICAL CONTEXTS

In the past there have been disagreements on how fast information can be learned and whether LTM can be formed after a single experience. Like in the examples mentioned above, one trial learning has been shown in several other invertebrate species: In the snail *Lymnaea stagnalis*, LTM is formed after a single trial in a foraging situation. The snail is able to

TABLE 1 | What to consider when testing insects for one trial learning.

Testing for one trial learning in insects

- Ecology (predictable vs. unpredictable environment; which cues are easy to learn)
- Life stage (larva vs. adult)
- Motivation of the insect (e.g., food deprived)
- Refine experimental set-ups
- Lab vs. field study

Learning salient information quickly should be highly adaptive for most insect species. When testing for one trial learning several aspects have to be considered.

associate the organic compound amyl acetate with a food reward. Interestingly, in this invertebrate LTM formation can only be blocked in a short time window 10 min to 1 h after training, which points to a very rapid memory consolidation process (Fulton et al., 2005). This is an important finding, since it means that LTMs can be formed very fast and consolidated quickly in order to ensure survival. This should be especially important when it comes to predator avoidance. We argue that learning the danger of a predator quickly and within a single trial could be highly adaptive for a species. That one trial learning of predator avoidance is actually possible has been shown in an ecologically relevant study by Henaut et al. (2014). The golden silk spider *Nephila clavipes* and the ectatomine ant *Ectatomma tuberculatum* are found in the same Neotropical habitat and are known to regularly encounter each other on the same plants (Figure 1). If an ant falls off a leave, the possibility is high that it gets trapped in the net of the arachnid. Since the ant possesses strong mandibles and a sting, this puts the spider in a dangerous situation. Experiments showed that if the spider is bitten by the ant only once, it will suppress its catching response and avoid this ant species in the future. If tested, it is also able to discriminate successfully between the ant and a non-threatening prey (fruit fly) item after a single trial. Similarly, the ant learns to escape the web of the spider more quickly if once entangled in it. It learns to cut the less sturdy threats of the web and gets out of the trap more efficiently (Henaut et al., 2014).

Also learning environmental cues that indicate valuable foraging or oviposition sites quickly can be key to survival and successful reproduction. When looking at insects in specific, we could show that the hawkmoth *Manduca sexta* is not only able to associate a neutral odor with a sugar reward after only one trial when visiting a flower (Adam et al., 2021), but it is further able to remember a suitable host plant for oviposition after only one visit to the leaf of this plant (Nataraj et al., 2021). The parasitic wasp *Lariophagus distinguendus* also easily learns to connect an artificial odor with the presence of its host (i.e., its oviposition site). As in the hawkmoth *Manduca sexta* and the above mentioned *Cotesia glomerata* only one trial is needed to learn where to oviposit (Collatz et al., 2006). Similarly, a single conditioning trial is enough for the parasitic wasps *Trichogramma evanescence* and *Nasonia vitripennis* to learn an odor that indicates the presence of their hosts. While memory retention does not vary in small and large conspecifics of *T. evanescence*, smaller wasps of *N. vitripennis* have lower levels of

memory retention than larger individuals, indicating cognitive costs in brain scaling (Van Der Woude et al., 2018). Despite fast retention of memory, learned information should, however, also be susceptible to fast alterations in case environmental factors change. Balderrama (1980) could show that the American cockroach (*Periplaneta americana*) is able to learn to reverse its odor preference within one trial. When tested, the cockroach shows no significant decay of this LTM over an interval of 1–7 days. This indicates that important memories are kept for long time periods if not a lifetime, but can be over-written with more salient information in case it proves adaptive.

DISCUSSION

In the past insect learning and memory has been underestimated. This was in part due to how learning and memory experiments were conducted under artificial lab conditions. Refining the experimental set-ups in the lab as well as designing research studies in an ecologically relevant framework has shown that insects are indeed able to learn salient olfactory information in as little as one trial. We argue that it should be highly adaptive for a species to learn olfactory cues indicating predators, food sources or oviposition sites quickly. Therefore, one trial

learning and quick LTM formation should be found in more insect species. We hypothesize that even the model organism *D. melanogaster* may be able to form long-term memories within one trial when tested in an ecologically relevant context. We think that considering the insects' ecology and motivation when designing experiments to test for one trial learning is key (Table 1). Also keeping in mind that some cues are easier to learn for one species than for another is important. These cues might even vary between life stages of the given species since insects can change their ecology quite drastically over their life span. In summary, one trial olfactory learning could be a general trait in insects and we look forward to more studies in this field.

AUTHOR CONTRIBUTIONS

EA: writing—original draft. BH and MK: writing—review and editing and supervision. BH: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by Max Planck Society.

REFERENCES

- Adam, E., Hansson, B. S., and Knaden, M. (2021). Moths sense but do not learn flower odors with their proboscis during flower investigation. *J. Exp. Biol.* 224:jeb242780. doi: 10.1242/jeb.242780
- Avarguès-Weber, A., and Giurfa, M. (2013). Conceptual learning by miniature brains. *Proc. R. Soc. B Biol. Sci.* 280:20131907. doi: 10.1098/rspb.2013.1907
- Balderrama, N. (1980). One trial learning in the American cockroach, *Periplaneta americana*. *J. Insect Physiol.* 26, 499–504. doi: 10.1016/0022-1910(80)90123-7
- Carey, A. F., and Carlson, J. R. (2011). Insect olfaction from model systems to disease control. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12987–12995. doi: 10.1073/pnas.1103472108
- Chittka, L., Giurfa, M., and Riffel, J. A. (2019). Editorial: the mechanisms of insect cognition. *Front. Psychol.* 10:2751. doi: 10.3389/fpsyg.2019.02751
- Cognigni, P., Felsenberg, J., and Waddell, S. (2018). Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*. *Curr. Opin. Neurobiol.* 49, 51–58. doi: 10.1016/j.conb.2017.12.002
- Collatz, J., Müller, C., and Steidle, J. L. (2006). Protein synthesis-dependent long-term memory induced by one single associative training trial in the parasitic wasp *Lariophagus distinguendus*. *Learn. Mem.* 13, 263–266. doi: 10.1101/lm.192506
- Davis, R. L. (2005). Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. *Annu. Rev. Neurosci.* 28, 275–302. doi: 10.1146/annurev.neuro.28.061604.135651
- Dezazzo, J., and Tully, T. (1995). Dissection of memory formation: from behavioral pharmacology to molecular genetics. *Trends Neurosci.* 18, 212–218. doi: 10.1016/0166-2236(95)93905-d
- Dion, E., Monteiro, A., and Nieberding, C. M. (2019). The role of learning on insect and spider sexual behaviors, sexual trait evolution, and speciation. *Front. Ecol. Evol.* 6:225. doi: 10.3389/fevo.2018.00225
- Dukas, R. (2008). Evolutionary biology of insect learning. *Annu. Rev. Entomol.* 53, 145–160. doi: 10.1146/annurev.ento.53.103106.093343
- Dunlap, A. S., and Stephens, D. W. (2014). Experimental evolution of prepared learning. *Proc. Nat. Acad. Sci.* 111, 11750–11755. doi: 10.1073/pnas.1404176111
- Dweck, H., Talross, G., Wang, W., and Carlson, J. (2021). Evolutionary shifts in taste coding in the fruit pest *Drosophila suzukii*. *Elife* 10:e64317. doi: 10.7554/eLife.64317
- Eschbach, C., Fushiki, A., Winding, M., Afonso, B., Andrade, I. V., Cocanougher, B. T., et al. (2021). Circuits for integrating learned and innate valences in the insect brain. *Elife* 10:e62567. doi: 10.7554/eLife.62567
- Fulton, D., Kemenes, I., Andrew, R. J., and Benjamin, P. R. (2005). A single time-window for protein synthesis-dependent long-term memory formation after one-trial appetitive conditioning. *Eur. J. Neurosci.* 21, 1347–1358. doi: 10.1111/j.1460-9568.2005.03970.x
- Giurfa, M. (2013). Cognition with few neurons: Higher-order learning in insects. *Trends Neurosci.* 36, 285–294. doi: 10.1016/j.tins.2012.12.011
- Giurfa, M. (2015). Learning and cognition in insects. *Wiley Interdiscip. Rev. Cogn. Sci.* 6, 383–395. doi: 10.1002/wcs.1348
- Giurfa, M., and Sandoz, J. C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* 19, 54–66. doi: 10.1101/lm.024711.111
- Güven-Ozkan, T., and Davis, R. L. (2014). Functional neuroanatomy of *Drosophila* olfactory memory formation. *Learn. Memory* 21, 519–526. doi: 10.1101/lm.034363.114
- Hammer, M., and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci.* 15, 1617–1630. doi: 10.1523/JNEUROSCI.15-03-01617.1995
- Haverkamp, A., and Smid, H. M. (2020). A neuronal arms race: the role of learning in parasitoid–host interactions. *Curr. Opin. Insect Sci.* 42, 47–54. doi: 10.1016/j.cois.2020.09.003
- Henaut, Y., Machkour-M'rabet, S., and Lachaud, J. P. (2014). The role of learning in risk-avoidance strategies during spider-ant interactions. *Anim. Cogn.* 17, 185–195. doi: 10.1007/s10071-013-0651-x
- Huber, R., and Knaden, M. (2018). Desert ants possess distinct memories for food and nest odors. *Proc. Natl. Acad. Sci. U.S.A.* 115, 10470–10474. doi: 10.1073/pnas.1809433115
- Inami, S., Sato, T., Kurata, Y., Suzuki, Y., Kitamoto, T., and Sakai, T. (2021). Consolidation and maintenance of long-term memory involve dual functions of the developmental regulator Apterous in clock neurons and mushroom bodies in the *Drosophila* brain. *PLoS Biol.* 19:e3001459. doi: 10.1371/journal.pbio.3001459
- Kahsai, L., and Zars, T. (2011). "Learning and memory in *drosophila*: behavior, genetics, and neural systems," in *International Review of Neurobiology*, ed N. Atkinson (London, UK: Academic Press), 139–167. doi: 10.1016/B978-0-12-387003-2.00006-9

- Keene, A. C., and Waddell, S. (2007). *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci.* 8, 341–354. doi: 10.1038/nrn2098
- Keleman, K., Vrontou, E., Krüttner, S., Yu, J. Y., Kurtovic-Kozaric, A., and Dickson, B. J. (2012). Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. *Nature* 489, 145–149. doi: 10.1038/nature11345
- Knaden, M., and Graham, P. (2016). The sensory ecology of ant navigation: From natural environments to neural mechanisms. *Annu. Rev. Entomol.* 61, 63–76. doi: 10.1146/annurev-ento-010715-023703
- Krashes, M. J., and Waddell, S. (2008). Rapid consolidation to a *radish* and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in *Drosophila*. *J. Neurosci.* 28, 3103–3113. doi: 10.1523/JNEUROSCI.5333-07.2008
- Lavery, T. M. (1980). The flower-visiting behaviour of bumble bees: floral complexity and learning. *Can. J. Zool.* 58, 1324–1335. doi: 10.1139/z80-184
- Leadbeater, E., and Chittka, L. (2007). Social learning in insects: From miniature brains to consensus building. *Curr. Biol.* 17, R703–R713. doi: 10.1016/j.cub.2007.06.012
- Lee, W.-P., Chiang, M.-H., Chang, L.-Y., Lee, J.-Y., Tsai, Y.-L., Chiu, T.-H., et al. (2020). Mushroom body subsets encode CREB2-dependent water-reward long-term memory in *Drosophila*. *PLoS Genet.* 16:e1008963. doi: 10.1371/journal.pgen.1008963
- Lee, W. P., Chiang, M. H., Chang, L. Y., Shyu, W. H., Chiu, T. H., Fu, T. F., et al. (2021). Serotonin signals modulate mushroom body output neurons for sustaining water-reward long-term memory in *Drosophila*. *Front. Cell Dev Biol.* 9:755574. doi: 10.3389/fcell.2021.755574
- Lihoreau, M., Dubois, T., Gomez-Moracho, T., Kraus, S., Monchanin, C., and Pasquaretta, C. (2019). “Chapter One - Putting the ecology back into insect cognition research,” in *Advances in Insect Physiology*, ed R. Jurenka (London, UK: Academic Press), 1–25. doi: 10.1016/bs.aiip.2019.08.002
- Lin, S., Oswald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. *Nat. Neurosci.* 17, 1536–1542. doi: 10.1038/nn.3827
- Menzel, R. (1999). Memory dynamics in the honeybee. *J. Comparat. Physiol. A* 185, 323–340. doi: 10.1007/s003590050392
- Menzel, R. (2012). The honeybee as a model for understanding the basis of cognition. *Nat. Rev. Neurosci.* 13, 758–768. doi: 10.1038/nrn3357
- Nataraj, N., Adam, E., Hansson, B. S., and Knaden, M. (2021). Host plant constancy in ovipositing *Manduca sexta*. *J. Chem. Ecol.* 47, 1042–1048. doi: 10.1007/s10886-021-01309-3
- Paoli, M., and Galizia, G. C. (2021). Olfactory coding in honeybees. *Cell Tissue Res.* 383, 35–58. doi: 10.1007/s00441-020-03385-5
- Papaj, D. R., and Prokopy, R. J. (1989). Ecological and evolutionary aspects of learning in phytophagous insects. *Annu. Rev. Entomol.* 34, 315–350. doi: 10.1146/annurev.en.34.010189.001531
- Reinhard, J., Srinivasan, M. V., and Zhang, S. (2004). Scent-triggered navigation in honeybees. *Nature* 427, 411–411. doi: 10.1038/427411a
- Smid, H. M., and Vet, L. E. M. (2016). The complexity of learning, memory and neural processes in an evolutionary ecological context. *Curr. Opin. Insect Sci.* 15, 61–69. doi: 10.1016/j.cois.2016.03.008
- Smid, H. M., Wang, G., Bukovinszky, T., Steidle, J. L. M., Bleeker, M. A. K., Van Loon, J. J. A., et al. (2007). Species-specific acquisition and consolidation of long-term memory in parasitic wasps. *Proc. R. Soc. B Biol. Sci.* 274, 1539–1546. doi: 10.1098/rspb.2007.0305
- Takeda, K. (1961). Classical conditioned response in the honey bee. *J. Insect Physiol.* 6, 168–179. doi: 10.1016/0022-1910(61)90060-9
- Tully, T., Preat, T., Boynton, S. C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79, 35–47. doi: 10.1016/0092-8674(94)90398-0
- Tully, T., and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comparat. Physiol. A* 157, 263–277. doi: 10.1007/BF01350033
- Van Der Woude, E., Huigens, M. E., and Smid, H. M. (2018). Differential effects of brain size on memory performance in parasitic wasps. *Anim. Behav.* 141, 57–66. doi: 10.1016/j.anbehav.2018.05.011
- Van Lenteren, J. C., and Bakker, K. (1975). Discrimination between parasitised and unparasitised hosts in the parasitic wasp *Pseudeucoila bochei*: a matter of learning. *Nature* 254, 417–419. doi: 10.1038/254417a0
- Villar, M. E., Marchal, P., Viola, H., and Giurfa, M. (2020). Redefining single-trial memories in the honeybee. *Cell Rep.* 30, 2603–2613.e3. doi: 10.1016/j.celrep.2020.01.086
- Weiglein, A., Gerstner, F., Mancini, N., Schleyer, M., and Gerber, B. (2019). One-trial learning in larval *Drosophila*. *Learn. Mem.* 26, 109–120. doi: 10.1101/lm.049106.118

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Adam, Hansson and Knaden. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Discussion

“Adaptability is the simple secret of survival.”

Jessica Hagedorn

Learning is the means to adapt to an ever changing environment. This not only holds true for humans, but also for tiny organisms such as insects. In my thesis, my aim was to discover more about olfactory learning in insects and in specific about olfactory learning in the context of foraging and oviposition in the tobacco hawkmoth (*Manduca sexta*):

My first aim was to determine whether the olfactory sensilla on the tip of the proboscis of *M. sexta* are involved in flower odor learning. The second aim was to find out if the tobacco hawkmoth is able to remember host plants it has previously oviposited on and if it will consecutively prefer these plants (i.e. show oviposition constancy). The third aim of the thesis was to create a comprehensive overview of one trial learning in insects. It was inspired by the first two manuscripts which both showed that *M. sexta* is a very fast learner. This stood in stark contrast with past literature that suggested that insects need multiple training trials that are spaced apart in time in order to learn. Therefore, I decided to search for similar findings in the field of insect learning and summarize what is known so far about the incredible olfactory learning capabilities of insects.

Discussion of The Context of Foraging (Manuscript I)

During foraging, the tobacco hawkmoth tracks the odor of the flower to its source, investigates the flower with the proboscis and finally inserts the proboscis to drink the nectar (Raguso and Willis, 2002). Since the moth hovers in mid-air during the whole process, reducing handling time of the flower is advantageous for the insect in order to conserve energy. Learning how to handle a flower (quickly) should therefore be highly adaptive.

In the first experiment of Manuscript I, I explored whether *M. sexta* is able to learn how to manipulate flowers more successfully over time – that is whether it becomes more efficient, so it will spend less time hovering at the flower while feeding. I tested the moths using a flower array with *Nicotiana attenuata* flowers that were fixed in the same upward angle to ensure comparability (see Figure 1 of Manuscript I). When allowing the moths to forage at the array, they indeed became more successful in handling the flowers over time (see Figure 6B of Manuscript I). Also, if the moths were successful during the first five flower manipulations, they explored more flowers in total (see Figure 6A of Manuscript I).

This increase in motivation is likely due to appetitive learning since the flowers contained their natural nectar volumes. Feeding on the nectar (US) would therefore have reinforced the behavior of flower investigation and motivated the moth to forage. This of course raises the question of what type of information (CS) the moth learns while it investigates the flower. Since the focus of this thesis is “olfactory learning”, I explored whether/how olfactory information is learned during this process:

To test odor learning in *M. sexta*, I designed an artificial flower that acted as a supernormal stimulus and allowed to contain the odor within the flower (see Figure 2 of Manuscript I). This was necessary in order to attract the moth to the artificial flower even in the absence of an innately attractive odor. Further, it allowed to test whether the olfactory sensilla on the tip of the moth’s proboscis described by Haverkamp et al. (2016) play any role in flower odor learning.

To establish whether *M. sexta* learns odors with its proboscis and/or the antennae only, I first had to determine the innate valence of the odor I wanted to use for testing. By presenting the moths with a choice between the test odor (linalool +/-) and an odor control (both emitted by separate odor valves within the wind tunnel, see Figure 3A of Manuscript I), I could confirm that the test odor was innately neutral. This was an important step since it meant that the test odor could be used as a CS for the learning experiments. In the next step, I trained the moths to associate the odor (CS) with a sugar reward (US) and in the last step, I again tested the odor valence (see Figure 3B and 3C of Manuscript I). An increase in attraction to the odor meant that *M. sexta* had learned the association between odor and sugar reward, choosing the test odor and the odor control equally often meant that the odor was not learnt. When the moths were able to perceive the odor with the antennae during training, there was a clear increase in attraction during the consecutive testing step (see Figure 7A of Manuscript I). This is consistent with previous findings that tobacco hawkmoths are able to learn odors using their antennae (Cook et al., 2020, Daly et al., 2001b, Daly and Smith, 2000, Riffell et al., 2008, Riffell et al., 2013). I could also show that one training trial was enough for the moths to learn the association between odor and reward. However, if the moths could only perceive the odor with the proboscis during odor conditioning training with the artificial flower, no increase of attraction was visible (see Figure 7B of Manuscript I). This of course raised the question whether learning odors with the proboscis is generally not possible or whether there is just no cross-talk between proboscis and the antennae.

I therefore decided to adjust the testing paradigm. Instead of offering a choice between two odor valves in the wind tunnel (which were perceived with the antennae), I provided a proboscis Y-maze within the artificial flower to test the proboscis in specific (see Figure 2C of Manuscript I). Hence, the moths were trained and tested with odor restricted to the proboscis only (see Figure 4 of Manuscript I). Again, no attraction to the odor was seen after the proboscis training (see Figure 7C of Manuscript I), indicating that there might be no learning with the proboscis.

However, since this was quite an artificial training/testing situation, I decided to go one step further and ask the same question in a more ecologically relevant manner that also allowed to test whether there is cross-talk between antennae and the proboscis. As mentioned before, the moth first flies up to a flower, then investigates it with the proboscis and only then inserts the proboscis into the flower (Raguso and Willis, 2002). That means that at first the moth might only be able to perceive the flower odor with the antennae, since the proboscis stays rolled up until the last moment when the moth starts to investigate the flower. Only then, the proboscis is unrolled and the sensilla at the tip of the proboscis might be exposed completely. Therefore, I created a training situation with odor on the in- and outside of the artificial flower (see Figure 5A of Manuscript I). Hence, the moth could smell the odor with the antennae and proboscis during the approach of the flower and foraging similarly as in a natural situation. If there was cross-talk between the antennae and the proboscis, this would mean that the moth would first learn the odor with the antennae and would later be able to choose it in the Y-maze with the proboscis.

However, when testing, the moths again did not show increased attraction to the odor (see Figure 7D of Manuscript I). That means that *M. sexta* does not seem to use the proboscis in any context of odor learning. Further, there seems to be no cross-talk between antennae and proboscis, since the odor that was learnt with the antennae was not selected more often/explored longer than the control odor with the proboscis in the Y-maze.

Hawkmoth Ecology and Odor Learning

How can these results be seen in an ecological or learning context? The answer may lie in the step by step sequence of the foraging behavior of the moth. *M. sexta* is able to track flower odors over 15 meters in order to forage (Raguso and Willis, 2005). That means it is able to smell the plant odor (CS) for quite some time before consuming the nectar (US). This could be considered as “forward pairing” of CS and US. The proboscis is, however, unrolled quite late (Raguso and Willis, 2002). Hence, the exposure to the odor when using the

proboscis is far shorter and could be considered as “simultaneous conditioning” rather than “forward pairing”. Experiments conducted on other animals such as Sprague Dawley rats (*Rattus norvegicus*), demonstrated that “simultaneous conditioning” is sufficient to form an association between CS and US in a fear conditioning paradigm. However, “forward pairing” was more effective and also allowed the rats to anticipate the US, which “simultaneous conditioning” did not (Barnet et al., 1991). This is especially interesting when seen in an ecological context for *M. sexta*: The flower odor should be predictive of the nectar reward in order for the moth to have a benefit of learning it. Therefore, learning an odor via the antennae compared to the proboscis might be advantageous. Also, hovering in front of the flower is highly energy consuming (Bartholomew and Casey, 1978) and it would cost a lot of energy to use the proboscis as a (singular) “nose” for flower odor learning. From an ecological standpoint, it would thus make more sense if the moth learned the flower odor mainly (or exclusively) with the antennae.

Instant Reward Increases Foraging Motivation

Before going into detail about what could be a possible function of the sensilla on the tip of the proboscis of *M. sexta*, I want to shortly discuss foraging motivation i.e. the motivation to explore flowers for a nectar reward. As mentioned above, the motivation to forage and investigate consecutive flowers increased in *M. sexta* when the moth was successfully foraging at a natural *N. attenuata* flower within the first five flower visits. If the moth was not able to access the nectar of the flowers within that time, I observed that it was more likely to give up early and cease foraging altogether (see Figure 6A, Manuscript I). I also observed a similar pattern at the artificial flower: If the flower did not contain a sugar reward during the first visits or the moth was not able to access the flower with the proboscis due to insufficient fine motor control, the moth would lose interest fast and would not return to the flower (data unpublished). This was also the reason why it was not possible to test the innate preference in the proboscis Y-maze before training the moth. Once it had thoroughly explored the artificial flower without receiving a sugar reward, it was not possible to motivate the moth to forage there again. Therefore, I simplified the paradigm and worked with an experimental and control group during these experiments (see Figure 4 and 5 of Manuscript I).

Both observations indicate that the ingestion of a nectar/sugar reward (US) is a key motivation for *M. sexta* to continue foraging. Similar results have been found in the honeybee (*Apis mellifera*). In this species the flow rate of the nectar determined the foraging effort of the bee – that is higher nectar flow rates increased the foraging motivation and effort (Giurfa,

1996). Furthermore, flowers that offer less nectar volume are abandoned more frequently by bumblebees (*Bombus impatiens*) and ignored completely if the flowers contain a lower sugar concentration than others (Cnaani et al., 2006). This indicates that the presence of a reward and its quality are important for continued foraging motivation among insects.

Is the Proboscis a Gustatory Organ for the Quality Assessment of the Flower?

What could be the role of the sensilla on the proboscis of *M. sexta* if they are not involved in olfactory learning? Haverkamp et al. (2016) could show that the innately attractive flower odor benzyl acetone (BA) emitted by *N. attenuata* increased foraging motivation in *M. sexta* compared to flowers that did not emit this odor. In the same study, the moths did not only spend more time exploring real *N. attenuata* flowers that emitted BA, but also investigated the proboscis Y-maze arm that contained BA longer than the control arm. Therefore, the sensilla might play a role in increasing initial foraging motivation in *M. sexta*. Even though to maintain foraging motivation over a prolonged period of time positive reinforcement through a reward such as nectar or sugar solution seems to be necessary.

Another possibility could be that the sensilla are used to assess the quality of the flower. In his thesis Haverkamp (2017) discusses the sensilla at the tip of the proboscis as possible “final control sensors” that allow to recognize the chemical profile of a flower. This of course raises the question of how the information of the proboscis is integrated in the central nervous system. Due to Haverkamp et al. (2016) the multiporous sensilla at the tip of the proboscis house three neurons out of which one is expressing the olfactory co-receptor (Orco), an indication of its olfactory function. Single sensillum recordings by the authors have shown that the sensillum responds to many different volatile compounds and most strongly to the innately attractive odor BA. The innately neutral odor I specifically selected for my experiments (linalool +/-; see Manuscript I) is among the compounds demonstrated by Haverkamp et al. (2016) eliciting a response that is about one third of the response to BA. Although in Haverkamp et al. (2016) the Orco positive sensillum responds to linalool during single sensillum recordings, my experiments showed that there is no baseline attraction to this odor when the proboscis is tested with the proboscis Y-maze (Manuscript I). This could mean that the difference in spike quantity translates into different behavioral output e.g. increasing the motivation to investigate a Y-maze arm containing an innately attractive odor while an innately neutral odor is not further explored.

Therefore, the question arises whether the olfactory input from the proboscis is processed similarly as olfactory input from the antennae – that means being integrated at the AL and

sent to higher brain centers such as the MB and LH (**Figure 3**) – or whether a local circuit within the subesophageal ganglion (SOG), a brain structure well known for gustatory processing (Miyazaki and Ito, 2010) is computing this odor information. In the malaria mosquito (*Anopheles gambiae*) odor information from the antennae and the maxillary palps is integrated at the AL and odor information from the labella of the proboscis is sent to glomeruli of the SOG (Riabinina et al., 2016). Exploring whether olfactory information is processed similarly in *M. sexta* could explain why there is a difference visible between the two “noses” of the insect, and why learning is possible with the antennae but not with the proboscis.

The question of how olfactory information from the proboscis is processed is also compelling when looking at olfaction versus gustation: In insects, primitive olfactory receptors like Orco and higher olfactory receptors (ORs) are an expansion of the gustatory receptor gene family (GRs) (Robertson, 2019, Robertson et al., 2003, Thoma et al., 2019), indicating that in terms of evolution, both senses might be closely linked. For example, gustatory neurons are able to detect odorants if they are genetically altered in *Drosophila melanogaster* to express odorant receptors (Hiroi et al., 2008). In this context, even though the neurons in the sensilla at the tip of the moth’s proboscis express the olfactory co-receptor, further investigation may validate a possible role of these neurons in rather gustatory function.

Discussion of The Context of Oviposition (Manuscript II)

The gravid female *M. sexta* moth chooses leaves for oviposition based on visual, olfactory and contact cues (Yamamoto et al., 1969). In Manuscript II my colleague and I wanted to know if previous experience with host plant leaves leads to learning and therefore influences consecutive oviposition choices. As in foraging decisions, showing “oviposition constancy” could potentially be adaptive for the insect.

The Ovipositor: Involved in Olfactory Host Leaf Learning?

M. sexta is known as both pollinator (adult) as well as herbivore (larva) of the plants *D. wrightii* and *N. attenuata* (Kessler and Baldwin, 2001, Kessler and Baldwin, 2006, Yamamoto and Fraenkel, 1960). The female moth oviposits on the leaves of these solanaceous plants where the larvae will later feed and develop (Kessler and Baldwin, 2001, Yamamoto and Fraenkel, 1960). In the first experiment of Manuscript II my colleague and I wanted to know whether the moth shows an innate preference for one of the two plants, so we would have a control baseline for the consecutive experiments (see Figure 1A of Manuscript II). Indeed, *M. sexta* oviposited more eggs on *D. wrightii* than on *N. attenuata* (see Figure 2 of Manuscript

II). This is in accordance with previous results from our department that also showed a clear oviposition preference for *D. wrightii* over *N. attenuata* (Späthe et al., 2013b). Since the moths in our study chose the plants equally often during the first contact (see Figure 2 of Manuscript II), this could mean that contact cues from the leaf itself have an influence on the oviposition decision.

This raises the question whether the perceived cues that determine the oviposition decision are mechanosensory, olfactory or gustatory in nature. As on the proboscis, the female moth possesses olfactory receptors on the ovipositor. Single sensillum recordings have shown that some of these sensilla react to odorants (Klinner et al., 2016). Again, it would be interesting to see how the information from the neurons housed in these sensilla is integrated in the central nervous system and whether the function of these neurons is rather olfactory or gustatory. Also, there is the question whether the olfactory sensilla of the ovipositor are involved in odor learning. Excluding the proboscis from this function (Manuscript I), I would hypothesize that the ovipositor also has a rather innate (gustatory?) than olfactory learning function.

One Trial and Prepared Learning in The Context of Oviposition

In the second experiment of Manuscript II, my colleague and I tested whether one experience with the leaf of either *D. wrightii* or *N. attenuata* is enough to shape the consecutive preference of the female. We could show that the moths indeed preferred leaves of the same plant after experiencing it once and chose it more often during their first choice. After the first choice, the moths also deposited more eggs on the familiar plant leaf than on the unfamiliar one (see Figure 2 of Manuscript II). The innate preference to oviposit on *D. wrightii* was additionally increased after learning. This increased preference was stable even on the consecutive day, indicating LTM formation. However, the preference for *N. attenuata* was lost on the next day (see Figure 2 of Manuscript II).

The question is why *M. sexta* innately prefers *D. wrightii* and shows an increased preference for this plant even on the next day if it has oviposited on it before. This stands in contrast with findings that the larvae of *M. sexta* actually accept *D. wrightii* less readily for feeding and perform less well on it in terms of growth compared with different *Nicotiana* species (Yamamoto and Fraenkel, 1960). One possible explanation could be a potential selection process in the lab for moths that innately prefer *D. wrightii* since these plants are used for egg collection over generations in the breeding colony. In accordance, long-term experiments on *D. melanogaster* flies could show that “prepared learning” (i.e. the capability to learn certain cues easier than others) can be traced to evolutionary selection (Dunlap and Stephens,

2014). In this study, the authors systematically manipulated the reliability of the pairing of an aversive cue (US) with an odor (CS) or a color (CS). After forty generations of evolution, the flies showed increased learning ability for the reliable cue (CS). A similar sensitization process may have happened for the lab population of *M. sexta* while learning *D. wrightii* as a reliable oviposition substrate over generations.

When looking at the behavior of oviposition as an appetitive (associative) learning experience, a possible CS could be visual, olfactory, gustatory or mechanosensory. However, what will be very interesting to find out is what actually acts as the US. Is it the process of egg deposition itself or are cues of the host plant leaf acting as US?

Motivation and Oviposition

In the last experiment of Manuscript II, my colleague and I asked whether past experience with a leaf would decrease the time the moth took to oviposit on consecutive leaves (i.e. increase the oviposition efficiency). Again, we saw differences between *N. attenuata* and *D. wrightii*. For *D. wrightii* the time decreased significantly while the time measured stayed the same for *N. attenuata* (see Figure 3 of Manuscript II). However, it is important to note, that for *N. attenuata* the oviposition efficiency was already high to begin with and moths took a short amount of time to fly up to the plant and oviposit on it from the start (also see Figure 3 of Manuscript II). A possible reason could be that *N. attenuata* and *D. wrightii* have different levels of odor emission: While *D. wrightii* foliage has a more subtle smell, *N. attenuata* is emitting a strong odor bouquet (Späthe et al., 2013a). This could have influenced the efficiency to locate the leaf, despite the fact that the leaf sizes were matched during the experiment to avoid leaf size as an influential factor.

On the one hand, the increase in oviposition efficiency we see for *D. wrightii* could reflect more proficiency in locating the leaf as well as less time spent investigating it before oviposition. On the other hand, it could mean that the motivation to oviposit in the moth is increased through the previous experience with a *D. wrightii* leaf. Similar as in foraging, conditioning with the (unknown) US could have increased oviposition motivation.

Discussion of One Trial Learning (Manuscript I-III)

In both Manuscripts I and II, I could show that (olfactory) learning is possible in *M. sexta* within just one trial. In Manuscript I, the moth learned to associate an innately neutral odor with a sugar reward by using its antennae. It still needs to be investigated how long this memory persists and whether it is similar like in other insects. For instance, ants (*Cataglyphis*

fortis) can retain odor information learned in one foraging trial for up to a lifetime (Huber and Knaden, 2018). In Manuscript II, moths retained information about a suitable oviposition site after one trial for (at least) one day. This stands in contrast with previous work on insects that show that multiple trials that are spaced apart in time are needed in order to form LTMs (Beck et al., 2000, Daly and Smith, 2000, DeZazzo and Tully, 1995, Menzel, 1999, Tully and Quinn, 1985). Therefore, I decided to conduct a literature search for similar findings that resulted in Manuscript III, a review about one trial olfactory learning in insects. It summarizes these findings and draws conclusions for future research on insect olfaction and learning.

How to Improve Studies on Olfactory Learning in Insects

For Manuscript III, I want to highlight the following key points:

1. Insects are able to learn (odor) information within one trial and this information can be retained up to a lifetime (Adam et al., 2021, Balderrama, 1980, Collatz et al., 2006, Henaut et al., 2014, Huber and Knaden, 2018, Nataraj et al., 2021, Takeda, 1961, van der Woude et al., 2018, Villar et al., 2020).
2. It should be highly adaptive for an insect to learn fast in order to avoid danger (Henaut et al., 2014) or to learn about important resources such as foraging (Adam et al., 2021, Huber and Knaden, 2018) or oviposition sites (Collatz et al., 2006, Nataraj et al., 2021).
3. In order for an insect to learn fast, the insect needs to be (highly) motivated to learn (Krashes and Waddell, 2008, Lee et al., 2020, Lin et al., 2014).
4. Some insects learn better than others and this can be explained by their ecology (Smid et al., 2007). If the environment is very predictable, LTMs are of advantage, if it is quite unpredictable plasticity in memory formation or active forgetting is more adaptive (Smid and Vet, 2016). These factors can even vary between the life stages of the insect.
5. An insect may have a predisposition to learn certain types of stimuli (CS) and might not be able to learn others quite as well (Dunlap and Stephens, 2014, Huber and Knaden, 2018). This predisposition to learn is termed “prepared learning” (Smid and Vet, 2016).
6. In order to test insect olfactory learning appropriately, experimental set-ups have to be well designed and sometimes need to be refined.
7. Testing the insect in an ecologically relevant context is important. Field studies may give more insights than artificial lab settings.

Setting the Results of Manuscript I & II into Perspective

In both contexts – foraging as well as oviposition – *M. sexta* was able to form associations very quickly (Manuscript I & II). This connects to point 1 of Manuscript III, where I discuss that also other insect species have been shown to have the ability to learn information very fast. Since both foraging as well as oviposition are crucial for the survival of the moth and its offspring, learning odor information rapidly and forming LTMs should be highly adaptive (see points 2 & 4 of Manuscript III). Especially, since both behaviors mostly involve the same plant species that can be reliably found in the habitat of *M. sexta* (Haverkamp et al., 2016, Kessler et al., 2010, Yamamoto and Fraenkel, 1960). What is important is that the nectar volume of the plant can actually influence oviposition itself. A study on the closely related *Manduca quinquemaculata* showed that oviposition increases significantly if the nectar volume of *D. wrightii* is artificially increased (Kessler, 2012). This raises the question whether the nectar gives information about the health of the plant (i.e. a healthy plant will produce more nectar) or acts as additional positive reinforcement (US) to increase the motivation of the moth (see point 3 of Manuscript III). Smith et al. (2018) found that *M. sexta* forages and oviposits at both *D. wrightii* and *Datura discolor*. Yet, while *D. wrightii* supports consecutive larval growth, *D. discolor* does not. The authors hypothesize that successful nectaring at *D. discolor* might lead the female moth to oviposit on the inferior host plant (Smith et al., 2018). This could indicate an increase in motivation after nectar ingestion rather than appropriate information gained about the health or quality of the plant. Since I also observed an increase of motivation to forage after successful nectaring in Manuscript III, this scenario seems very likely and should be investigated in the future.

Conclusion

Learning is the inherent ability of an organism to adapt to its environment. This holds true for insects and humans alike. The tobacco hawkmoth *M. sexta* is able to learn pertinent (olfactory) information in as little as one trial, both in the context of foraging as well as oviposition. The olfactory sensilla on the tip of the proboscis do, however, not seem to play a role in the learning process and might serve a rather innate function. In the context of oviposition, *M. sexta* is able to learn host plant cues and show “oviposition constancy”. Still, there seems to be a predisposition to learn certain host plant cues better than others. To set these results into perspective and to create a comprehensive overview over research on one trial learning, I wrote Manuscript III entitled “Fast Learners: One Trial Olfactory Learning in Insects”. I hope it will also inspire other scientists to investigate more about the incredible olfactory learning abilities of insects.

Summary

Most organisms rely on learning to be able to adapt to an ever changing environment. Even tiny insects have evolved the ability to learn despite the small size of their brain and their comparably short life spans. But what exactly do insects learn and how do they learn it? The aim of this thesis was to understand more about learning in insects and in specific about olfactory learning during foraging and oviposition. To investigate this topic, I used the tobacco hawkmoth (*Manduca sexta*) as model organism and conducted behavioral experiments in the wind tunnel of our institute. Its advantage is that it offers the possibility to run experiments in a natural as possible setting while allowing for standardized experimental conditions.

The first aim of the thesis was to determine whether the proboscis of *M. sexta* is involved in olfactory learning (Manuscript I). I investigated this question with the help of an artificial flower that I especially designed for the study. It allowed to stimulate and test the proboscis with an odor without stimulating the antennae at the same time. This was necessary to be able to test the capabilities of the proboscis. The results of Manuscript I suggest that olfactory learning is not possible with the tip of the proboscis and that it might either serve an innate olfactory or gustatory function for the quality assessment of the flower.

The second aim of this thesis was to establish whether *M. sexta* is able to learn host plants for oviposition and will consecutively show “oviposition constancy”. In Manuscript II, my colleague and I could indeed show that this is the case. There seems to be, however, an innate predisposition to what plant cues are learned more easily than others.

Since in both Manuscript I and Manuscript II *M. sexta* showed surprisingly fast learning skills, this inspired me to run a comprehensive literature search for similar learning abilities in other insect species. The result of this research is Manuscript III, a review about one trial learning in insects. In this review I summarize the findings of this still rather small research field. I hope this review will spark the interest of more researchers to investigate the olfactory learning abilities of insects in an ecologically relevant context.

Zusammenfassung

Die meisten Organismen dieser Erde sind auf die Fähigkeit zu lernen angewiesen, um sich erfolgreich an ihre Umwelt anpassen zu können. Das gilt selbst für kleine Insekten. Trotz ihres vergleichbar winzigen Gehirns und ihrer kurzen Lebensspanne haben sie die Fähigkeit zu lernen entwickelt. Aber was lernen Insekten eigentlich? Das Ziel dieser Dissertation war es, mehr über Lernen bei Insekten heraus zu finden – im speziellen über olfaktorisches Lernen bei der Futtersuche und Eiablage. Um das Thema näher zu erforschen, habe ich mit dem nachtaktiven Tabakschwärmer (*Manduca sexta*) als Modellorganismus gearbeitet und Verhaltensversuche im Windtunnel des Institutes durchgeführt. Der Windtunnel hat den Vorteil, dass man Experimente unter naturnahen, jedoch standardisierten Bedingungen durchführen kann.

Das erste Ziel dieser Dissertation war, fest zu stellen, ob die olfaktorischen Sensillen, die auf der Spitze des Saugrüssels von *M. sexta* zu finden sind, eine Rolle beim Lernen von Blütendüften spielen (Manuskript I). Ich habe diese Frage mit Hilfe einer künstlichen Blume, die speziell für diese Studie gefertigt wurde, erforscht. Die artifizielle Blume hat es ermöglicht den Saugrüssel mit einem Geruch zu stimulieren ohne gleichzeitig die Antennen zu stimulieren. Das war nötig, um den Einfluss des Saugrüssels auf das Lernen erfolgreich zu testen. Die Resultate von Manuskript I zeigen, dass die Sensillen auf der Spitze des Saugrüssels sehr wahrscheinlich keine Rolle beim Erlernen von Blütendüften spielen. Eine angeborene olfaktorische oder gustatorische Funktion zur Prüfung der Blütenqualität könnte hingegen denkbar sein. Das zweite Ziel der Dissertation war, heraus zu finden, ob *M. sexta* Wirtspflanzen zur Eiablage erlernen kann und nach einem erfolgreichen Besuch zur selben Wirtspflanze zurückkehrt, das heißt Wirtspflanzen-Stetigkeit zeigt. In Manuskript II konnten meine Kollegin und ich zeigen, dass das tatsächlich der Fall ist. Es scheint bei *M. sexta* jedoch eine angeborene Prädisposition zu geben, so dass manche Wirtspflanzen leichter gelernt und besser erinnert werden als andere. Nachdem *M. sexta* sowohl in Manuskript I als auch II eine überraschend rasche Lernfähigkeit gezeigt hat, habe ich eine umfassende Literaturrecherche zu schnellen Lernfähigkeiten in Insekten durchgeführt. Die Ergebnisse dieser Recherche habe ich in einem Review über One Trial Lernen in Insekten zusammengefasst (Manuskript III). Es detailliert die Ergebnisse dieses noch ziemlich kleinen Forschungsfeldes. Ich hoffe, dass ich mit dem Review das Interesse anderer Naturwissenschaftler wecken kann, mehr über die faszinierenden olfaktorischen Lernfähigkeiten von Insekten zu erforschen.

References

- ADAM, E., HANSSON, B. S. & KNADEN, M. 2021. Moths sense but do not learn flower odors with their proboscis during flower investigation. *Journal of Experimental Biology*, 224, jeb242780.
- ADAM, E., HANSSON, B. S. & KNADEN, M. 2022. Fast learners: One trial olfactory learning in insects. *Frontiers in Ecology and Evolution*, 10, 876596.
- ALARCÓN, R., DAVIDOWITZ, G. & BRONSTEIN, J. L. 2008. Nectar usage in a southern Arizona hawkmoth community. *Ecological Entomology*, 33, 503-509.
- ALLMANN, S., SPÄTHE, A., BISCH-KNADEN, S., KALLENBACH, M., REINECKE, A., SACHSE, S., BALDWIN, I. T. & HANSSON, B. S. 2013. Feeding-induced rearrangement of green leaf volatiles reduces moth oviposition. *eLife*, 2, e00421.
- ASO, Y., HATTORI, D., YU, Y., JOHNSTON, R. M., IYER, N. A., NGO, T.-T. B., DIONNE, H., ABBOTT, L. F., AXEL, R., TANIMOTO, H. & RUBIN, G. M. 2014. The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife*, 3, e04577.
- BALDERRAMA, N. 1980. One trial learning in the American cockroach, *Periplaneta americana*. *Journal of Insect Physiology*, 26, 499-504.
- BARNET, R. C., ARNOLD, H. M. & MILLER, R. R. 1991. Simultaneous conditioning demonstrated in second-order conditioning: Evidence for similar associative structure in forward and simultaneous conditioning. *Learning and Motivation*, 22, 253-268.
- BARTHOLOMEW, G. A. & CASEY, T. M. 1978. Oxygen-consumption of moths during rest, pre-flight warm-up, and flight in relation to body size and wing morphology. *Journal of Experimental Biology*, 76, 11-25.
- BECK, C. D. O., SCHROEDER, B. & DAVIS, R. L. 2000. Learning performance of normal and mutant *Drosophila* after repeated conditioning trials with discrete stimuli. *Journal of Neuroscience*, 20, 2944-2953.
- BISCH-KNADEN, S., DAHAKE, A., SACHSE, S., KNADEN, M. & HANSSON, B. S. 2018. Spatial representation of feeding and oviposition odors in the brain of a hawkmoth. *Cell Reports*, 22, 2482-2492.
- BOTO, T., STAHL, A. & TOMCHIK, S. M. 2020. Cellular and circuit mechanisms of olfactory associative learning in *Drosophila*. *Journal of Neurogenetics*, 34, 36-46.
- BUEHLMANN, C., GRAHAM, P., HANSSON, B. S. & KNADEN, M. 2014. Desert ants locate food by combining high sensitivity to food odors with extensive crosswind runs. *Current Biology*, 24, 960-964.
- BUEHLMANN, C., GRAHAM, P., HANSSON, B. S. & KNADEN, M. 2015. Desert ants use olfactory scenes for navigation. *Animal Behaviour*, 106, 99-105.
- BUSTO, G. U., CERVANTES-SANDOVAL, I. & DAVIS, R. L. 2010. Olfactory learning in *Drosophila*. *Physiology*, 25, 338-346.
- CAREY, A. F. & CARLSON, J. R. 2011. Insect olfaction from model systems to disease control. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 12987-12995.

- CHITTKA, L., THOMSON, J. D. & WASER, N. M. 1999. Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften*, 86, 361-377.
- CNAANI, J., THOMSON, J. D. & PAPA, D. R. 2006. Flower choice and learning in foraging bumblebees: Effects of variation in nectar volume and concentration. *Ethology*, 112, 278-285.
- COLLATZ, J., MULLER, C. & STEIDLE, J. L. M. 2006. Protein synthesis-dependent long-term memory induced by one single associative training trial in the parasitic wasp *Lariophagus distinguendus*. *Learning & Memory*, 13, 263-266.
- COOK, B., HAVERKAMP, A., HANSSON, B. S., ROULSTON, T., LERDAU, M. & KNADEN, M. 2020. Pollination in the anthropocene: a moth can learn ozone-altered floral blends. *Journal of Chemical Ecology*, 46, 987-996.
- D'ETTORRE, P., DEISIG, N. & SANDOZ, J.-C. 2017. Decoding ants' olfactory system sheds light on the evolution of social communication. *Proceedings of the National Academy of Sciences*, 114, 8911-8913.
- DALY, K. C., CHANDRA, S., DURTSCHI, M. L. & SMITH, B. H. 2001a. The generalization of an olfactory-based conditioned response reveals unique but overlapping odour representations in the moth *Manduca sexta*. *Journal of Experimental Biology*, 204, 3085-3095.
- DALY, K. C., DURTSCHI, M. L. & SMITH, B. H. 2001b. Olfactory-based discrimination learning in the moth, *Manduca sexta*. *Journal of Insect Physiology*, 47, 375-384.
- DALY, K. C. & SMITH, B. H. 2000. Associative olfactory learning in the moth *Manduca sexta*. *Journal of Experimental Biology*, 203, 2025-2038.
- DEZAZZO, J. & TULLY, T. 1995. Dissection of memory formation: From behavioral pharmacology to molecular genetics. *Trends in Neurosciences*, 18, 212-218.
- DOLAN, M.-J., FRECHTER, S., BATES, A. S., DAN, C., HUOVIALA, P., ROBERTS, R. J. V., SCHLEGEL, P., DHAWAN, S., TABANO, R., DIONNE, H., CHRISTOFOROU, C., CLOSE, K., SUTCLIFFE, B., GIULIANI, B., LI, F., COSTA, M., IHRKE, G., MEISSNER, G. W., BOCK, D. D., ASO, Y., RUBIN, G. M. & JEFFERIS, G. S. X. E. 2019. Neurogenetic dissection of the *Drosophila* lateral horn reveals major outputs, diverse behavioural functions, and interactions with the mushroom body. *eLife*, 8, e43079.
- DUKAS, R. 2008. Evolutionary biology of insect learning. *Annual Review of Entomology*, 53, 145-160.
- DUNLAP, A. S. & STEPHENS, D. W. 2014. Experimental evolution of prepared learning. *Proceedings of the National Academy of Sciences*, 111, 11750.
- ESCHBACH, C., FUSHIKI, A., WINDING, M., AFONSO, B., ANDRADE, I. V., COCANOUGH, B. T., EICHLER, K., GEPNER, R., SI, G., VALDES-ALEMAN, J., FETTER, R. D., GERSHOW, M., JEFFERIS, G. S. X. E., SAMUEL, A. D. T., TRUMAN, J. W., CARDONA, A. & ZLATIĆ, M. 2021. Circuits for integrating learned and innate valences in the insect brain. *eLife*, 10, e62567.
- FABRE, J.-H. 1918. *The wonders of instinct*, New York, Century Company, 10, 322.
- FELSENBERG, J., PLATH, J. A., LORANG, S., MORGENSTERN, L. & EISENHARDT, D. 2014. Short- and long-term memories formed upon backward conditioning in honeybees (*Apis mellifera*). *Learning & Memory*, 21, 37-45.

- GIURFA, M. 1996. Movement patterns of honeybee foragers: Motivation and decision rules dependent on the rate of reward. *Behaviour*, 133, 579-596.
- GIURFA, M. 2013. Cognition with few neurons: Higher-order learning in insects. *Trends in Neurosciences*, 36, 285-294.
- GOYRET, J., MARKWELL, P. M. & RAGUSO, R. A. 2007. The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *Journal of Experimental Biology*, 210, 1398-405.
- GOYRET, J. & YUAN, M. L. 2015. Influence of ambient illumination on the use of olfactory and visual signals by a nocturnal hawkmoth during close-range foraging. *Integrative and Comparative Biology*, 55, 486-494.
- HALLEM, E. A. & CARLSON, J. R. 2006. Coding of odors by a receptor repertoire. *Cell*, 125, 143-160.
- HAMMER, M. & MENZEL, R. 1995. Learning and memory in the honeybee. *Journal of Neuroscience*, 15, 1617-1630.
- HANSON, F. E. & DETHIER, V. G. 1973. Role of gustation and olfaction in food plant discrimination in the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology*, 19, 1019-1031.
- HAVERKAMP, A. 2017. *The chemosensory ecology of a foraging hawkmoth*. Doctoral thesis, Friedrich-Schiller-University, 121.
- HAVERKAMP, A., YON, F., KEESEY, I. W., MISSBACH, C., KOENIG, C., HANSSON, B. S., BALDWIN, I. T., KNADEN, M. & KESSLER, D. 2016. Hawkmoths evaluate scenting flowers with the tip of their proboscis. *eLife*, 5, e15039.
- HENAUT, Y., MACHKOUR-M'RABET, S. & LACHAUD, J. P. 2014. The role of learning in risk-avoidance strategies during spider-ant interactions. *Animal Cognition*, 17, 185-195.
- HIROI, M., TANIMURA, T. & MARION-POLL, F. 2008. Hedonic taste in *Drosophila* revealed by olfactory receptors expressed in taste neurons. *Plos One*, 3, e2610.
- HOMBERG, U., CHRISTENSEN, T. A. & HILDEBRAND, J. G. 1989. Structure and function of the deutocerebrum in insects. *Annual Review of Entomology*, 34, 477-501.
- HOWLETT, N., DAUBER, K. L., SHUKLA, A., MORTON, B., GLENDINNING, J. I., BRENT, E., GLEASON, C., ISLAM, F., IZQUIERDO, D., SANGHAVI, S., AFROZ, A., ASLAM, A., BARBARO, M., BLUTSTEIN, R., BOROVKA, M., DESIRE, B., ELIKHIS, A., FAN, Q., HOFFMAN, K., HUANG, A., KEEFE, D., LOPATIN, S., MILLER, S., PATEL, P., RIZZINI, D., ROBINSON, A., ROKINS, K., TURLIK, A. & MANSFIELD, J. H. 2012. Identification of chemosensory receptor genes in *Manduca sexta* and knockdown by RNA interference. *BMC Genomics*, 13, 211.
- HUBER, R. & KNADEN, M. 2018. Desert ants possess distinct memories for food and nest odors. *Proceedings of the National Academy of Sciences of the United States of America*, 115, 10470-10474.
- KAHSAL, L. & ZARS, T. 2011. Learning and memory in *Drosophila*: Behavior, genetics, and neural systems. In: ATKINSON, N. (ed.) *International Review of Neurobiology*. London: Academic Press, 139-167.

- KAISLING, K.-E. 2019. Responses of insect olfactory neurons to single pheromone molecules. In: PICIMBON, J.-F. (ed.) *Olfactory concepts of insect control - Alternative to insecticides: Volume 2*. Cham, Switzerland: Springer International Publishing, 1-27.
- KESSLER, A. & BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, 291, 2141-2144.
- KESSLER, D. 2012. Context dependency of nectar reward-guided oviposition. *Entomologia Experimentalis et Applicata*, 144, 112-122.
- KESSLER, D. & BALDWIN, I. T. 2006. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *The Plant Journal*, 49, 840-854.
- KESSLER, D., DIEZEL, C. & BALDWIN, I. T. 2010. Changing pollinators as a means of escaping herbivores. *Current Biology*, 20, 237-242.
- KLINNER, C. F., KÖNIG, C., MISSBACH, C., WERCKENTHIN, A., DALY, K. C., BISCH-KNADEN, S., STENGL, M., HANSSON, B. S. & GROßE-WILDE, E. 2016. Functional Olfactory Sensory Neurons Housed in Olfactory Sensilla on the Ovipositor of the Hawkmoth *Manduca sexta*. *Frontiers in Ecology and Evolution*, 4, 130.
- KRASHES, M. J. & WADDELL, S. 2008. Rapid consolidation to a *radish* and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in *Drosophila*. *Journal of Neuroscience*, 28, 3103-3113.
- KYMRE, J. H., LIU, X., IAN, E., BERGE, C. N., WANG, G., BERG, B. G., ZHAO, X. & CHU, X. 2021. Distinct protocerebral neuropils associated with attractive and aversive female-produced odorants in the male moth brain. *eLife*, 10, e65683.
- LANDOLT, P. J. 1997. Sex attractant and aggregation pheromones of male phytophagous insects. *American Entomologist*, 43, 12-22.
- LEE, W.-P., CHIANG, M.-H., CHANG, L.-Y., LEE, J.-Y., TSAI, Y.-L., CHIU, T.-H., CHIANG, H.-C., FU, T.-F., WU, T. & WU, C.-L. 2020. Mushroom body subsets encode CREB2-dependent water-reward long-term memory in *Drosophila*. *Plos Genetics*, 16, e1008963.
- LIHOREAU, M., DUBOIS, T., GOMEZ-MORACHO, T., KRAUS, S., MONCHANIN, C. & PASQUARETTA, C. 2019. Chapter One - Putting the ecology back into insect cognition research. In: JURENKA, R. (ed.) *Advances in Insect Physiology*. London: Academic Press, 1-25.
- LIN, S., OWALD, D., CHANDRA, V., TALBOT, C., HUETTEROTH, W. & WADDELL, S. 2014. Neural correlates of water reward in thirsty *Drosophila*. *Nature Neuroscience*, 17, 1536-1542.
- LIU, C., PLAÇAIS, P.-Y., YAMAGATA, N., PFEIFFER, B. D., ASO, Y., FRIEDRICH, A. B., SIWANOWICZ, I., RUBIN, G. M., PREAT, T. & TANIMOTO, H. 2012. A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature*, 488, 512-516.
- MAHONEY, W. J. & AYRES, J. J. B. 1976. One-trial simultaneous and backward fear conditioning as reflected in conditioned suppression of licking in rats. *Animal Learning & Behavior*, 4, 357-362.
- MAO, Z. & DAVIS, R. L. 2009. Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Frontiers in Neural Circuits*, 3, 5.

- MASSE, N. Y., TURNER, G. C. & JEFFERIS, G. S. X. E. 2009. Olfactory information processing in *Drosophila*. *Current Biology*, 19, R700-R713.
- MASSON, C. & MUSTAPARTA, H. 1990. Chemical information processing in the olfactory system of insects. *Physiological Reviews*, 70, 199-245.
- MECHABER, W. L., CAPALDO, C. T. & HILDEBRAND, J. G. 2002. Behavioral responses of adult female tobacco hornworms, *Manduca sexta*, to hostplant volatiles change with age and mating status. *Journal of Insect Science*, 2, 5.
- MENDA, G., BAR, H. Y., ARTHUR, B. J., RIVLIN, P. K., WYTTEBACH, R. A., STRAWDERMAN, R. L. & HOY, R. R. 2011. Classical conditioning through auditory stimuli in *Drosophila*: methods and models. *The Journal of Experimental Biology*, 214, 2864-2870.
- MENZEL, R. 1999. Memory dynamics in the honeybee. *Journal of Comparative Physiology A*, 185, 323-340.
- MIYAZAKI, T. & ITO, K. 2010. Neural Architecture of the Primary Gustatory Center of *Drosophila melanogaster* Visualized With GAL4 and LexA Enhancer-Trap Systems. *Journal of Comparative Neurology*, 518, 4147-4181.
- NATARAJ, N., ADAM, E., HANSSON, B. S. & KNADEN, M. 2021. Host plant constancy in ovipositing *Manduca sexta*. *Journal of Chemical Ecology*, 47, 1042-1048.
- PAPAJ, D. R. & PROKOPY, R. J. 1989. Ecological and evolutionary aspects of learning in phytophagous insects. *Annual Review of Entomology*, 34, 315-350.
- RAGUSO, R. A. & WILLIS, M. A. 2002. Synergy between visual and olfactory cues in nectar feeding by naïve hawkmoths, *Manduca sexta*. *Animal Behaviour*, 64, 685-695.
- RAGUSO, R. A. & WILLIS, M. A. 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Animal Behaviour*, 69, 407-418.
- REISENMAN, C. E., RIFFELL, J. A. & HILDEBRAND, J. G. 2009. Neuroethology of oviposition behavior in the moth *Manduca sexta*. *Annals of the New York Academy of Sciences*, 1170, 462-467.
- RENOU, M. & ANTON, S. 2020. Insect olfactory communication in a complex and changing world. *Current Opinion in Insect Science*, 42, 1-7.
- RIABININA, O., TASK, D., MARR, E., LIN, C.-C., ALFORD, R., O'BROCHTA, D. A. & POTTER, C. J. 2016. Organization of olfactory centres in the malaria mosquito *Anopheles gambiae*. *Nature Communications*, 7, 13010.
- RIFFELL, J. A., ALARCÓN, R., ABRELL, L., DAVIDOWITZ, G., BRONSTEIN, J. L. & HILDEBRAND, J. G. 2008. Behavioral consequences of innate preferences and olfactory learning in hawkmoth-flower interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 3404-3409.
- RIFFELL, J. A., LEI, H., ABRELL, L. & HILDEBRAND, J. G. 2013. Neural basis of a pollinator's buffet: olfactory specialization and learning in *Manduca sexta*. *Science*, 339, 200-204.
- ROBERTSON, H. M. 2019. Molecular evolution of the major arthropod chemoreceptor gene families. *Annual Review of Entomology*, 64, 227-242.

- ROBERTSON, H. M., WARR, C. G. & CARLSON, J. R. 2003. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 100, 14537-14542.
- SMID, H. M. & VET, L. E. M. 2016. The complexity of learning, memory and neural processes in an evolutionary ecological context. *Current Opinion in Insect Science*, 15, 61-69.
- SMID, H. M., WANG, G., BUKOVINSZKY, T., STEIDLE, J. L. M., BLEEKER, M. A. K., VAN LOON, J. J. A. & VET, L. E. M. 2007. Species-specific acquisition and consolidation of long-term memory in parasitic wasps. *Proceedings of the Royal Society B: Biological Sciences*, 274, 1539-1546.
- SMITH, G. P., JOHNSON, C. A., DAVIDOWITZ, G. & BRONSTEIN, J. L. 2018. Linkages between nectaring and oviposition preferences of *Manduca sexta* on two co-blooming *Datura* species in the Sonoran Desert. *Ecological Entomology*, 43, 85-92.
- SPÄTHE, A., REINECKE, A., HAVERKAMP, A., HANSSON, B. S. & KNADEN, M. 2013a. Host plant odors represent immiscible information entities - Blend composition and concentration matter in hawkmoths. *Plos One*, 8, e77135.
- SPÄTHE, A., REINECKE, A., OLSSON, S. B., KESAVAN, S., KNADEN, M. & HANSSON, B. S. 2013b. Plant species- and status-specific odorant blends guide oviposition choice in the moth *Manduca sexta*. *Chemical Senses*, 38, 147-159.
- STOCKER, R. F., LIENHARD, M. C., BORST, A. & FISCHBACH, K. F. 1990. Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. *Cell and Tissue Research*, 262, 9-34.
- TAKEDA, K. 1961. Classical conditioned response in the honey bee. *Journal of Insect Physiology*, 6, 168-179.
- TEMPEL, B. L., BONINI, N., DAWSON, D. R. & QUINN, W. G. 1983. Reward Learning in Normal and Mutant *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 80, 1482-1486.
- THOMA, M., MISSBACH, C., JORDAN, M. D., GROSSE-WILDE, E., NEWCOMB, R. D. & HANSSON, B. S. 2019. Transcriptome surveys in silverfish suggest a multistep origin of the insect odorant receptor gene family. *Frontiers in Ecology and Evolution*, 7, 281.
- TICHENOR, L. H., SEIGLER, D. S. & WEI, L. 1981. Oviposition responses of *Manduca sexta* to solanaceous volatile components. *Transactions of the Illinois State Academy of Science*, 74, 35-41.
- TULLY, T., PREAT, T., BOYNTON, S. C. & DEL VECCHIO, M. 1994. Genetic dissection of consolidated memory in *Drosophila*. *Cell*, 79, 35-47.
- TULLY, T. & QUINN, W. G. 1985. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *Journal of Comparative Physiology A*, 157, 263-277.
- VAN DER WOUDE, E., HUIGENS, M. E. & SMID, H. M. 2018. Differential effects of brain size on memory performance in parasitic wasps. *Animal Behaviour*, 141, 57-66.
- VAN LENTEREN, J. C. & BAKKER, K. 1975. Discrimination between parasitised and unparasitised hosts in the parasitic wasp *Pseudeucoila bochei*: a matter of learning. *Nature*, 254, 417-419.
- VILLAR, M. E., MARCHAL, P., VIOLA, H. & GIURFA, M. 2020. Redefining single-trial memories in the honeybee. *Cell Reports*, 30, 2603-2613.

- WASER, N. M. 1986. Flower constancy: Definition, cause, and measurement. *The American Naturalist*, 127, 593-603.
- YAMAMOTO, R. T. & FRAENKEL, G. S. 1960. The specificity of the tobacco hornworm, *Protoparce sexta*, to solanaceous plants. *Annals of the Entomological Society of America*, 53, 503-507.
- YAMAMOTO, R. T., JENKINS, R. Y. & MCCLUSKY, R. K. 1969. Factors determining the selection of plants for oviposition by the tobacco hornworm *Manduca sexta*. *Entomologia Experimentalis et Applicata*, 12, 504-508.
- ZHANG, J., BISCH-KNADEN, S., FANDINO, R. A., YAN, S., OBIERO, G. F., GROSSE-WILDE, E., HANSSON, B. S. & KNADEN, M. 2019. The olfactory coreceptor IR8a governs larval feces-mediated competition avoidance in a hawkmoth. *Proceedings of the National Academy of Sciences*, 116, 21828-21833.
- ZHANG, J., KOMAIL RAZA, S. A., WEI, Z., KEESEY, I. W., PARKER, A. L., FEISTEL, F., CHEN, J., CASSAU, S., FANDINO, R. A., GROSSE-WILDE, E., DONG, S., KINGSOLVER, J., GERSHENZON, J., KNADEN, M. & HANSSON, B. S. 2022. Competing beetles attract egg laying in a hawkmoth. *Current Biology*, 32, 861-869.
- ZHAO, Z. & MCBRIDE, C. S. 2020. Evolution of olfactory circuits in insects. *Journal of Comparative Physiology A*, 206, 353-367.

Declaration of Independent Assignment

I, the author of this thesis, am aware of the applicable doctoral examination regulations.

I declare in accordance with the conferral of the degree of doctor from the Faculty of Biological Sciences of the Friedrich-Schiller University Jena that the submitted thesis was written by me with only the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of manuscripts are listed as co-authors of the respective manuscript.

I was not assisted by a consultant for doctorate theses. Third parties have neither directly nor indirectly received monetary benefits from me for work related to the content of the submitted dissertation.

The thesis has not been previously submitted whether to the Friedrich-Schiller University Jena or to any other university.

.....

Place, Date

.....

Elisabeth Adam, MSc

Acknowledgements

<i>Bill & Susanne</i>	for making the department such a welcoming place to work at ☺!!
<i>Markus</i>	for being a great “Doktorvater” and making working together such an enjoyable time. I had a lot of fun writing the review with you!!
<i>Silke</i>	for letting me sneak back into the “ <i>Drosophila</i> -world” and join the imaging meetings. I learned a lot!
<i>Swetlana</i>	for helping me with ALL my questions regarding field trip organization, conferences, office supplies and whatever else I had on my mind.
<i>Holger</i>	for being my official supervisor at the FSU. Thank you for your support at our TAC meetings and your great advice concerning the thesis!
<i>Alex & Richard</i>	for introducing <i>Manduca sexta</i> to me and showing me the ropes at the wind tunnel. You two guys are amazing!
<i>Daniel</i>	for accepting me as „one of the workshop guys” and helping me realize all my crazy ideas. You’re the best person to contact if one needs to organize a field trip within a short period of time!!
<i>Julius</i>	for a great field trip to Greece together. I couldn’t have asked for a better master student to be in the field with.
<i>Saskia †</i>	for organizing the best “Lange Nacht der Wissenschaft” together, ever!! I truly miss you and your smile.
<i>Veit</i>	for explaining the microscopes to me and quick trouble shooting. Your dry sense of humor is the best cure for malfunctioning microscopes!
<i>All TAs</i>	especially Sascha, Silke T. and Roland for making the work with <i>Manduca</i> and <i>Drosophila</i> possible. You guys are the heroes of the lab!
<i>Alberto (AMC)</i>	for exciting scientific discussions, always being there for me, making me fight and supporting me through the tough times. Sometimes a strong woman needs a strong man to have her back!
<i>Kalpana</i>	for all the crazy things we did together ☺. I don’t think I need to say more. You’re my little sister from another country.
<i>Karen</i>	for coming into my life unexpected and bringing your whimsical energy. Going to the gym without you will not be the same!
<i>Melissa</i>	for enjoying the same sense of humor. You know what I mean ;)!

<i>Vignesh</i>	best office mate ever! I'm sorry I kicked you out of the office on the first day hahaha. To my defense: It was high alert Covid time.
<i>Nandita</i>	one of the strongest women I know. Thank you for your irreplaceable pep talks and making me smile.
<i>Angie</i>	for showing me how to climb and get over my fears of height. You're the best climbing instructor 😊!
<i>Ana & Diego</i>	for amazing dinners together. Diego you're a great cook and Ana you know how to entertain!
<i>Jin & Shuwei</i>	for making the office a fun place to work at and for the most entertaining movie experience ever hahaha! Also for teaching me the improved <i>Manduca sexta</i> egg injection protocol.
<i>Megha</i>	for your dry sense of humor that always makes me laugh and being a great teammate at the <i>Manduca</i> keeping.
<i>Devasena</i>	for making <i>Drosophila</i> oviposition assays a fun pastime. We rocked those cages!!
<i>Steve</i>	for explaining to me why some man's dream is another man's horror.
<i>Sarah</i>	for organizing all the fun movie nights, hat making events and epic Halloween parties and introducing us into PhD life!
<i>Mohammed</i>	for making sure JC was taking place regularly. I always liked discussing papers with you and running into you in the kitchen.
<i>Ian</i>	for finding my license plate when it fell off my car due to Austrian-German license plate holder incompatibilities.
<i>Yusuke</i>	for being the first Japanese to tell me "Just say no!". I hope you do not get kicked out of your country for that ;).
<i>Sin</i>	for being the best PhD representative and coming up with all kinds of ideas for making PhD life more interesting.
<i>Soma, Ali B. & Anjana</i>	for accepting me as a fellow "Indian". I feel honored 😊!
<i>Venkatesh</i>	for reminding me of my soft side. I sometimes forget that I have one.
<i>Sabine B.</i>	for all the entertaining stories about your life in Namibia and making working in the same space such a smooth endeavor.
<i>Lorena</i>	for all the advice concerning handing in the thesis and a great afternoon of ice skating.

<i>Regina & Lydia</i>	my fellow food truck regulars. I will miss having burgers with you girls!
<i>Rolando</i>	for showing me how to use the equipment at the fitness studio. I still laugh about your smirk when you kept repeating “You’ll have to lower the weights now...”.
<i>Sudeshna</i>	for the most epic yoga laughing fit. Yoga is not the same without you!
<i>Benjamin</i>	B before A and C next to F. (Sorry, I could not do that in my thesis, it would have hurt me just as much as you.)
<i>Further thanks</i>	goes to Sonja, Jürgen, Dieter, Florencia, Ali, Marília, Eric, Pramit, Xingcong, Ahmed, Eleftherios, Nanji, Julio, Marina, Hetan, and all other people who made the Hansson Department such a great place to work at during my time as a doctoral researcher.
<i>Christian</i>	“Du scho wieda!” (“Oh no, not you again!”)

Appendix

FORMULAR 2

Manuskript Nr. I

Kurzreferenz: Adam et al., 2021; J. Exp. Biol.

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

Abbildung(en) # 6 & 7	<input checked="" type="checkbox"/>	100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	<input type="checkbox"/>	0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	<input type="checkbox"/>	Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: _____% Kurzbeschreibung des Beitrages: (z. B. „Abbildungsteile a, d und f“ oder „Auswertung der Daten“ etc)

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in (Mitglied der Fakultät)

FORMULAR 2

Manuskript Nr. II

Kurzreferenz: Nataraj et al., 2021; J. Chem. Ecol.

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

Abbildung(en) # 2 & 3	<input type="checkbox"/> 100 % (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
	<input type="checkbox"/> 0 % (die in dieser Abbildung wiedergegebenen Daten basieren ausschließlich auf Arbeiten anderer Koautoren)
	<input checked="" type="checkbox"/> Etwaiger Beitrag des Doktoranden / der Doktorandin zur Abbildung: <u>20</u> % Kurzbeschreibung des Beitrages: <i>Konzeption der Experimente, Datenauswertung, Training der Erstautorin die Windtunnel Experimente durch zu führen</i>

Unterschrift Kandidat/-in

Unterschrift Betreuer/-in (Mitglied der Fakultät)