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"Punishing Effect of *Rhodiola rosea* in larval *Drosophila melanogaster*"

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Abstract

The aim of this thesis is to investigate the reinforcing effects that *R. rosea* has in larval *D. melanogaster*, an established study case for associative learning. *R. rosea* root material was previously known to have a rewarding effect in a one-odor learning paradigm in *D. melanogaster* larvae. Here it is shown that, in addition, this plant material has as punishing effect. Given that a compound that can mediate the rewarding effects of *R. rosea* has recently been identified, it was the aim of this thesis to identify the chemical compound(s) responsible for the punishing effect. Towards this end a bio-assay guided fractionation with activity-correlation analysis was performed. Each fractionation step was tested in the one-odor learning paradigm for effectiveness. In the end a single compound could not be identified. The results suggest, however, that the punishing and the rewarding effects of *R. rosea* are not mediated by the very same compound. The second part of this thesis focuses on the neuronal circuitry underlying the punishing effect of *R. rosea* in *D. melanogaster* larvae. Transgenic manipulation of the larval mushroom body, a brain area known for memory formation, was performed using the UAS/ Gal4 system. UAS-Kir_{GFP}/ OK107-*Gal4* animals showed impairments in *R. rosea* extract punishment memory. However, experiments for innate responses towards odor and *R. rosea* extract showed differences to genetic control animals, such that these differences might partially account for the observed punishment memory defects.

Keywords

Rhodiola rosea, *Drosophila melanogaster*, larva, olfactory learning, punishment memory

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List of Abbreviations

ACORA	activity-correlation analysis
AM	odor *n-amyl acetate*
BSSG	*β-siterol-β-D-glucoside*
CS	Canton Special (*D. melanogaster* wildtype strain), conditioned stimulus
CR	conditioned response
DO	dorsal organ
E1-E5	ethyl acetate subfractions
EM	no odor (“empty”)
EtOH	ethanol
F1	first generation
FAE-20	*ferulic acid eicosyl ester*
FASII	fasciclin II
Gal4	yeast transcription activator protein Gal4
GFP	green fluorescent protein
H1-H4	heptane subfractions
HTS	high throughput screening
KC	Kenyon cells
Kir	inward rectifying potassium channel
KW	Kruska-Wallis-Test
LAL	larval antennal lobe
LH	lateral horn
MB	mushroom body
MS	mass spectrometry
MWU	Mann-Whitney-U-Test
N	number of animals
NGS	normal goat serum
OK107-*Gal4*	driver line of Gal4 expression pattern in the larval mushroom bodies
ORN	olfactory neurons
OSS	one-sample sign test
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Triton X-100
PI	performance index
PN	projection neuron
$Pref_{AM}$	odor preference
$Pref_{AM}$(AM+)	odor preference when AM was reinforced during training
$Pref_{AM}$(EM+)	odor preference when EM was reinforced during training
$Pref_{RH}$	*Rhodiola rosea* extract taste preference
SOG	subesophageal target region
UAS	upstream activating site
UAS-Kir_{GFP}	transgenic construct coding for hyperpolarizing K^+-channel, GFP tagged
US	unconditioned stimulus
WT	wildtype
X	gene of interest
+	reinforcement; wildtype allel in transgenetic experiments

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Introduction

1 Background of *Rhodiola rosea*

R. rosea is a plant native to the arctic regions of Europe, Asia and North America and was historically used for the treatment of a broad range of illnesses, including psychiatric diseases (historically Linné 1749; Panossian et al. 2010). Indeed, a processed extract of *R. rosea* can be used today as prescription-free herbal medication for depression, fatigue (Olsson et al. 2009) and anxiety (Cropley et al. 2015).

1.1 Use of *Rhodiola rosea* in humans

During the last 20 years, scientific publications on *R. rosea* in various fields have yielded interesting findings. In clinical trials antidepressant, memory-enhancing and fatigue-relieving effects in healthy volunteers (Darbinyan et al. 2000) and patients with mild depression (Darbinyan et al. 2007) were described for *R. rosea*. Mao et al. (2015) even discuss that *R. rosea* might be more beneficial due to its low side effects profile in mild depression patients and therefore have a better patient compliance compared to conventional treatment with antidepressant sertraline. Clinical studies so far showed no or only mild side effects of *R. rosea* treatment in humans, which would make it a pharmacologically safe possibility (Hung et al. 2011). Van Diermen et al. (2009) found a *R. rosea* induced inhibition of monoamine-oxidase A, one of the target enzymes in depression medication, as a possible explanation for the antidepressant effects. Other clinical trials in healthy objects showed an anxiety-decreasing and a mood-increasing effect of *R. rosea*-extract (Cropley et al. 2015). Also influences on the cortisol axis in humans have been reported (Darbinyan et al. 2007). Other studies show enhancement in mental as well as physical performance (Hung et al. 2011) or immunomodulatory functions such as antiviral (Ahmed et al. 2015) and antioxidative (Furmanowa et al. 1998) effects, as well as mood and pleasure increasing properties during physical exercise (Duncan & Clarke 2014). Further studies suggest neuroprotective effects of *R. rosea* in Alzheimer's disease by influencing some of the key pathophysiologies, like decreasing neuroinflammation and oxidative stress (Lee et al. 2013; Nabavi et al. 2016). However, the beneficial effects of *R. rosea* must be reviewed with caution as there are also published studies that showed an opposite effect, such as Punja et al. (2014), who reported that a *R. rosea* treated group actually showed increasing fatigue-related symptoms. However, official reports by the European Medicine Agency (Langer 2012) and the National Institutes of Health of the USA (NCCIH 2016) remain skeptical of the effectiveness of *R. rosea* treatments due to a lack of high quality clinical trials.

1.2 Investigations in animals and microorganisms

In animals and microorganisms, *R. rosea* has shown life-span increasing effects in *C. elegans* (Wiegant et al. 2009), *D. melanogaster* (Schriner et al. 2013) and yeast cells (Bayliak & Lushchak 2011). *R. rosea* extract was found effective for anti-inflammation and analgesia in rats (Doncheva et al., 2013). In an induced stress and depression rat model comparable to that of classical antidepressant drugs *R. rosea* extract showed antistress and antidepressant effects (Mattioli et al. 2009). Influences on serotonin (Mannucci et al. 2012) levels in the central nervous system and cortisol production (Yang et al. 2014) are discussed to be responsible for this effect in animals (Amsterdam & Panossian 2016). Furthermore antipsychotic-like effects of the *R. rosea* extract used in this thesis have been found as assayed through increased prepulse inhibition in rats and mice (Coors et al. 2019).

Related to this thesis, memory-enhancing (Michels et al. 2018) and rewarding (Michels et al. in press) effects of *R. rosea* and its compounds in *D. melanogaster* larvae were found. These findings will be further discussed and referenced in the course of this thesis.

1.3 Compounds found in *Rhodiola rosea*

Different chemical components in *R. rosea* could be identified. Ingredients that can confer the observed bioactivity were used in continuing studies about *R. Rosea*. Major chemical classes found in *R. rosea* are phenylpropanoid derivatives (e.g. rosavin) and phenylethanoid derivatives (e.g. salidroside, tyrosol), which were suggested to be responsible for the antidepressant and anti-fatigue effects (Amsterdam & Panossian 2016; Panossian et al. 2010; Darbinyan et al. 2000). Other chemical classes include flavonoids and phenolic acids, which reportedly confer antioxidant, antiinflammatory and cell-protective effects (Raj et al. 2010, Nabavi et al. 2016). Of note, although salidroside and rosavin are characteristic for *R. rosea* and their percentages in extract preparations are used for quality control, the question whether these substances are the bioactive compounds remains not fully answered (Mattioli et al. 2009).

Recently, Michels et al. (2018) and Michels et al. (in press) could identify the bioactive ferulic acid eicosyl ester (FAE-20) from *R. rosea* root material. FAE-20 shows memory enhancement in both *D. melanogaster* larvae and adult flies, as well as an improvement of

long-term memory in honeybees and an enhancement of hippocampus-dependent fear memory in mice. Interestingly, FAE-20 was capable to reduce memory impairments in a genetically altered flies with increased levels of the presynaptic *bruchpilot* protein. *Bruchpilot* is involved in a *D. melanogaster* model for Alzheimer's disease (Huang et al. 2013).

It is important to note that effects in studies can differ with the kind of *R. rosea* plant material (crude root, extract or single compound) used in the experiments. In general natural products and their metabolite profiles underlie some variability due to natural differences in several samples of the same plant by weather and environmental influences (Amsterdam & Panossian 2016) as well as different methods of plant production and processing (Wang et al. 2004; Yuliana et al. 2011). Therefore, it is of great interest to evaluate single bioactive compound(s) and their effects in controlled studies in order to obtain reproducible results that allow for drug development.

2 Larval *Drosophila melanogaster* as model organism for learning behavior

A long history of research in adult *D. melanogaster*, going back to the first description of a defined mutation in a fly by T.H. Morgan (1916) more than 100 years ago, established a rich knowledge about its brain anatomy, behavior, molecular processing and genetics. Enabled by a broad set of experimental possibilities, in particular of genetic manipulation, *D. melanogaster* was established as a key model organism in the biological and biomedical sciences, including research into associative olfactory memory (Tully & Quinn 1985; Heisenberg 2003; Modi et al. 2020). Larval *D. melanogaster* research, although fairly recent, appears to in no way be less suitable for research as compared to its adult counterpart with advantages like lower cell number of neuronal circuits (with a total number of "only" 10,000-15,000 neurons (Scott et al. 2001, Bose et al. 2015)), a translucent body, and easy handling and convenient availability of animals. As discussed in the following section, this also holds true for the study of the simple forms of memory in the larvae.

2.1 Associative learning in *Drosophila melanogaster* larvae

A larval olfactory tastant learning experiment has been first introduced by Scherer et al. (2003) as a two-group, reciprocal training with two odors and testing of individual animals. It was subsequently adapted to an one-odor paradigm (Saumweber et al. 2011) with en masse

testing (Neuser et al. 2005). It is modeled after a classical conditioning experiment in adult *D. melanogaster* flies by Quinn et al. (1974) where the animals are trained to associate an odor with an electric shock punishment (unconditioned stimulus – US) making the odor an conditioned stimulus (CS). As larvae spend a major part of their life feeding, gustatory reinforcers as US lead to robust results in associative learning (Diegelmann et al. 2013). Both reward and punishment memory paradigms with tastants have been established in larvae. Specifically, they show attraction to odors associated in a previous training with sweet and nutritious substances such as fructose, and aversion to odors associated with bitter compounds like quinine or with high concentration salt (Niewalda et al. 2008; Gerber et al. 2009). Larvae can also associate odors with reinforcers of other modalities, like electric shock (Heisenberg et al. 1985) or substrate vibration (Eschbach et al. 2011).

For the present thesis, it is important that although the animals are able to form a memory trace during training, this memory trace is not necessarily leading to observable learned behavior during the test, because the larvae compare the situation during the test with what they learned during training. Only if that comparison "promises a gain" from retrieving the memory earned behavior is expressed (Gerber & Hendel 2006; Schleyer et al. 2011). That is, in the appetitive case (e.g. odor-fructose learning) the animals would track down the odor in search of the associated positive reinforcer (in the example fructose) only if they are tested in the absence of that reinforcer. If tested in its presence, no memory is behaviorally expressed because nothing would be gained from doing so. In other words, it is adaptive to terminate search once the sought-for item is found. For the aversive case (e.g. odor-quinine learning) it is the other way around. The animals show avoidance of the odor as an escape behavior of the negative reinforcer (in this case quinine) only if they are tested in its presence. If tested in its absence, the larvae would not be exposed to a "bad situation" and therefore have no reason to escape. This might be likened to the case of humans knowing the meaning of an emergency exit sign, but refraining from using it unless there is a dangerous situation like a fire. Interestingly, Schleyer et al. (2015) showed that larvae memorize both the value (how "good" or "bad") and the quality (which substance) of the reinforcer used in training, revealing an unexpected complexity of their memory system. Adding to this complexity, sodium chlorine can act as positive or negative reinforcer, depending on its concentration (Niewalda et al. 2008; also see Toshima et al. 2019 for a similar observation for a 20 amino acid mixture), an observation fitting to our own experience that "a grain of salt" improves any dish, while dishes with too much salt are regarded as spoiled and are avoided.

These odor-taste learning experiments can easily be performed due to the convenient availability of large quanities of animals, and because very little special equipment and training of the experimenter are required (Diegelmann et al. 2013; Gerber & Stocker 2007). Together with the possibilities for genetic manipulation (also see section 2.3 below), this establishes larval *D. melanogaster* as a versatile insect model system for understanding the mechanisms of associative learning and memory.

2.2. Neuronal mechanisms of memory formation in *Drosophila melanogaster* larvae

2.2.1 Olfactory and gustatory pathways

The olfactory and gustatory pathways of *D. melanogaster* larvae have been studied in some detail (Python & Stocker 2002; for reviews see: Gerber & Stocker 2007; Vosshall & Stocker 2007; Gerber et al. 2009; Benton et al., 2009). They have three external and three internal chemosensory organs, all of which transmit gustatory information with the help of neurons expression receptors of the *Gr* and *Ir* gene families. In contrast, only the so-called dorsal organ (DO) also includes 21 olfactory neurons (ORNs) expressing receptor molecules of the *Or* gene family to serve olfactory function. The cell bodies of sensory neurons can be found in the ganglion of the respective external sensory organ. Olfactory afferents form synapses in glomerulus structures, similar to the olfactory bulb in vertebrates in the larval antennal lobe (LAL) with local interneurons contributing to coding of odor patterns and projection neurons (PNs) sending information to the mushroom body (MB) calyx and the lateral horn (LH) for higher processing with a combined divergence–convergence connectivity in the MB before connecting to motor centers. Signals from the LH provide a direct pathway to motor output, leading to experimentally naive olfactory behavior, whereas the MB detour is open to modulation of that behavior by integrating other environmental influences. Gustatory signals go to the subesophageal target region (SOG) which provides a direct connection between taste and motor centers, primarily bypassing "higher" processing, but also gives rise to octopamine- and dopaminergic interneurons that signal towards the MB.

D. melanogaster, both adult and larva, can be viewed as a model system for studying olfactory and taste processing, as they possess similarities to the mammalian chemosensory system, like *Or* expression or glomerular convergence, but at a much lower cell number (Python & Stocker 2002), allowing investigating influences on olfactory behavior in the larval case on a single-cell level (Gerber & Stocker 2007). Larval chemosensation possesses an

advantage to adult flies because of fewer neurons involved. Even though their peripheral chemosensory systems differ, they share the same principle of central connectivity (Vosshall & Stocker 2007). Adults, on the other hand, have to execute a much more complex behavior to survive than its larval counterpart, which must be reflected in its more complex neuronal circuit (Vosshall & Stocker 2007; Gerber et al. 2009). However, it is important to keep in mind that *D. melanogaster* and vertebrate chemosensory systems also show striking differences, especially in the taste pathway such that findings in *D. melanogaster* cannot be taken for granted in higher organisms.

2.2.2 Mushroom body

The mushroom body (MB) is a higher brain center found in both hemispheres of insects such as honeybee, cricket, cockroach and *D. melanogaster*. MB of adult *D. melanogaster* flies and larvae are organized in a similar way with cell numbers differing. Kenyon cells (KC) are the MB intrinsic cells whose neuropils make up the structure of the MB.

Olfactory PNs of the LAL terminate to glomeruli of the larval MB calyx and form synapses with MB γ neurons, which are embryonic and larval-born (Marin et al. 2005). This means olfactory information diverges and converges onto about 600 MB neurons found in third larval instar (Lee et al. 1999; Vosshall & Stocker 2007) depending if they form uni- or multiglomerular connections (Gerber & Stocker 2007). For comparison, adult flies have about 2500 MB neurons. Gustatory PNs signal gustatory reinforcing input more indirectly via aminergic MB input neurons to the MB as the general gustatory pathway connects the SOG directly with motor output centers (Gerber et al. 2009).

The KCs act as a coincidence detector for odor and reinforce signals reaching the MB making it the “seat of a memory trace for odours” (Heisenberg et al. 1985; Heisenberg 2003). Thus memory formation in the MB leads to modulations in MB output neurons signaling to motor centers and leading to observable changes olfactory behavior (Thum & Gerber 2019; Modi et al. 2020). Aversive gustatory signals are mediated via dopaminergic input to the MB (Schroll et al. 2006) and appetitive signals via octopaminergic (Schwaerzel et al. 2003) as well as dopaminergic input (Burke et al. 2012; Rohwedder et al. 2016).

Flies with defective MB show impairments in certain memory types (olfactory, spatial, courtship), whereas other memory types and other properties like locomotion seem unaffected

(Joiner & Griffith 2000; Zars 2000). Heisenberg (2003) summarized that the MB function besides olfactory learning processing in larval and adult *D. melanogaster* also includes "decision-like processes" like spontaneous walking activity of context processing in visual learning paradigms.

2.3 The UAS/Gal4 System: A genetic toolbox for exploring behavior

The genetic information of *D. melanogaster* is organized in four chromosomes. Sequencing led to the identification of about 13,600 genes (Adams et al. 2000) establishing the basis for targeted genetic research. Brand and Perrimon (1993) were the first to describe the binary UAS/Gal4 system allowing targeted gene expression in a specific tissue. *Gal4* is a yeast gene coding for a transcription factor that by itself induces no phenotype in *D. melanogaster* (Fischer et al. 1988; Duffy 2002). If expressed, the Gal4 transcription factor binds to an Upstream Activating Sequence (UAS) and thereby can induce transcription of whatever downstream gene, including any gene of interest (for better understanding marked with "X" in Fig. 2) cloned behind it by the experimenter. Transgenic flies that carry either only the construct for Gal4 inserted into their genome or only the UAS-X construct for the effector gene X do therefore not produce the effector protein X. When crossing a homozygous *Gal4* strain (so-called driver) to a homozygous UAS-X strain (so-called effector because the protein X mediates the experimentally intended effect), their F1 offspring carries both transgenic constructs, thereby producing the effector protein X in the pattern corresponding to the expression of Gal4. In many cases, *Gal4* is cloned by the experimenter behind a specific enhancer sequence of the *D. melanogaster* genome, leading to Gal4 expression in only those cells in which this particular enhancer sequence is active. This dependence of Gal4 expression on the enhancer located in its vicinity is the basis for the temporal and spatial diversity of transgene expression through different Gal4 lines. *D. melanogaster* research in the past decades has produced large collections and associated databases of different driver- and effector strains offering opportunities to visualize neuronal morphology, connectivity and activity, and manipulate the function of almost any neuron of interest to determine the impact of such manipulation on behavior (Jones 2009).

When testing transgenic animals in a behavioral assay of associative learning, it should be avoided to attribute an observed effect directly to a deficit in learning ability when in fact the transgenic manipulation could have led to defects in processing of the to-be-associated stimuli

or compromised faculties of locomotion (Heisenberg 2003). Therefore, it has been established to test untrained, experimentally naive animals for their naive responses towards the stimuli they would have to associate in the training. In the case of the experiments of the present thesis, this would be the behavior towards the odor (CS) and the tastant (US), in both the experimental genotype and its genetic controls (Gerber & Stocker 2007).

3. Natural products and identifying new bioactive compounds

Natural products (NP) are chemicals, mostly secondary metabolites of plants or microorganisms, that can be used as resources for the discovery of new leads in future pharmacological developments. A historically impressive example is the discovery of the antibiotic penicillin from the fungus *Penicillium* (Fleming 1929). Between 2005 and 2010, 19 drugs were approved which are the outcome of NP-based research (Mishra & Tiwari 2011). Even though NP-based research has been eclipsed by the focus of pharmaceutical companies on, as is hoped, faster methods such as high throughput screening (HTS) of synthetic libraries, NP-based research poses unique opportunities for drug development (Butler et al. 2014).

3.1 Bio-assay guided fractionation

A bio-assay guided isolation/fractionation tries to identify the bioactive compound(s) from any complex mixture by testing for efficacy in a biological paradigm, e.g. antibacterial screening by measuring bacteria-free areola. For that purpose, a crude extract is prepared which is purified into different fractions. Chromatography can be used to divide all ingredients by differences e.g. in their polarity or molecule size, as modern methods proved to be very efficient in the analysis of NPs. Separation can also result from use of electrophoresis or other techniques (Yang et al. 2001; Weller 2012, Wu et al. 2013). Bioactivity testing takes place after the extract preparation as well as after the fractionation steps to identify the bioactive fraction(s) that contain the compound(s) of interest. Bioactive fractions are further purified into subfractions and iteratively tested for their bioactivity. This process is continued until a candidate bioactive substance can be suggested that can then be structurally characterized by e.g. mass spectrometry (MS) (Wu et al. 2013). This approach has the advantage that instead of “blindly” isolating different chemical substances and then testing them, it is a targeted method, which only focuses on chemical mixtures that show bioactivity.

However, it is a very time- and material-consuming method and during each step, the previously seen effect might be lost e.g. if multiple synergistically active substances are separated. Dereplication, detecting already known compounds, might also be a scientifically interesting result, yet obviously is lacking innovation potential for drug development (Wu et al. 2013). To bypass the repeated fractionation, an activity-correlation-analysis (ACORA) can be used, which correlates metabolic profiles of contained molecules of the fractions, detected e.g. with combined MS, with their bioactivity results. It is assumed that bioactivity is directly proportional to the concentration of the bioactive compound or rather the peak intensity in MS analysis (Michels 2011, Hielscher-Michael et al. 2016) The result of such a correlation is a relatively short ”hit list” of peaks representing potential molecules, which show a statistically significant relation to the investigated bioactivity. These can then be isolated or synthesized for validation of bioactivity.

4 Possible pharmacological applications

This thesis aims to identify the punishing effector(s) of *R. rosea* in larvae of *D. melanogaster*. If successful, why would such a substance be interesting or useful? If the identified compounds were specifically active in insects, this might open the door to identifying insect repellents, with obvious utility for the management of insect disease vectors. For example, Malaria is an infectious disease transmitted by the female *Anopheles* mosquito as a vector. It poses a continuing danger of millions of people: In 2018, it was responsible for 405 000 deaths worldwide (WHO 2019). Vector-control, either by insecticide-treated mosquito nets or room sprays, is one of the key aims in primary disease prevention. However, an increasing number of resistances against currently used insecticides (WHO 2019) and side effects in humans who have come into contact with the insecticides (Ribas-Fitó et al. 2006; Bouwman et al. 2011) make it desirable to develop alternative, safe substances the *Anopheles* mosquito is repelled by. Obviously, the safety of such applications to humans would require further pharmacological and pharmacokinetic profiling, like toxicology and clinical testing. In recent developments, novel approaches to control infectious diseases were shown by identifying compounds altering the biting behavior of dengue fever transmitting *Aedes aegypti* mosquitoes (Duvall et al. 2019). Identifying the essential neuronal mechanisms underlying their biting behavior might indeed pose prospects of further insect manipulation. Approaching the issue from a different angle, Matthews et al. (2019) were able to show egg-laying behavioral changes in *Aedes aegypti* mediated by sensory neurons expressing a specific channel, suggesting that finding drugs

targeting this channel might offer new pharmacological possibilities for treating tropical infectious diseases.

The same principle would hold regarding the control of insect agricultural pests as recently covered by the media like the 2020 plague of locusts in eastern Africa, threatening the food harvest of the local population. Application in pest control in conventional agriculture yielding no human side effects might also be a possible use of novel drugs targeting insect behavior modulation.

Although these scenarios are highly speculative, they offer ideas to utilize larval *D. melanogaster* avoidance behavior or reinforcement learning in relation to *R. rosea* or other plants in a medical entomology context.

Methods

1 From *Rhodiola rosea* root to candidate substance(s): Bio-assay guided fractionation

To identify the bioactive compound(s) in *R. rosea* root, the bio-assay guided fractionation method (Fig. 1) was carried out as described in detail in Michels et al. (in press). In brief, at first an alcoholic extract was prepared out of 1.565 kg of *R. rosea* dried root material (Eveline24.de online shop). In the first fractionation step the *R. rosea* extract was treated with four solvents that differ in polarity (water, butanol, ethyl acetate, and heptane) to separate the compounds into four fractions according to their polarity. The effective ethyl acetate- and heptane fractions were then used to generate five ethyl acetate- and four heptane subfractions, all dissolved in ethanol (100 %). By the statistical ACORA method (Michels 2011) the behavioral data of the subfractions was correlated with the concentration and presence of the compounds detected in these subfractions. Through a combination of mass spectrometry and nuclear magnetic resonance chemically characterization of the subfractions would be possible to yield candidate compounds that show the punishing effect. Punishment memory scores in fractions and subfractions would be correlated with analytical mass signal intensities with the help of the Spearman rank correlation.

R. rosea preparations were stored in a fridge at 4 °C. All preparative and analytical steps of this bio-assay guided fractionation and the associated statistical analyses were kindly contributed by L. Wessjohann and K. Franke, Leibniz Institute of Plant Biochemistry (IPB), Halle. For a more detailed description of fractionation steps and activity correlation, please refer to Michels et al. (in press).

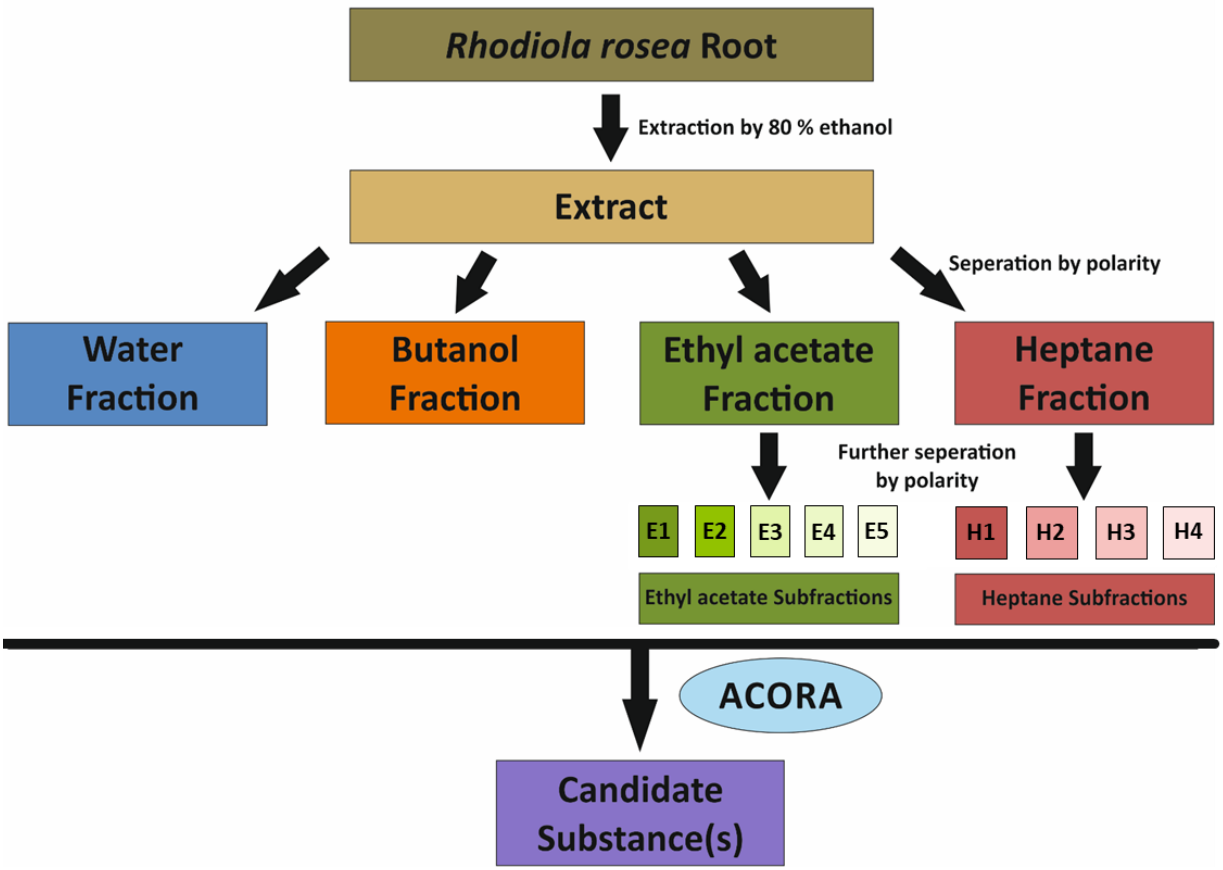

Fig. 1: Bio-assay guided fractionation to identify bioactive punishing compound(s) found in *Rhodiola rosea* root.
The bio-assay guided fractionation is a method to split up all contained ingredients in *R. rosea* to identify bioactive compound(s). Each step is then tested for effectiveness in punishment learning in *D. melanogaster* larvae. First, an alcoholic extract was prepared out of *R. rosea* dried root material. In the first fractionation step the *R. rosea* extract was treated with four solvents that differ in polarity (water, butanol, ethyl acetate, and heptane) to separate the compounds into four fractions depending on their polarity. The effective ethyl acetate- and heptane fractions were then used to generate five ethyl acetate (E1- 5) and four heptane (H1- 4) subfractions, all dissolved in ethanol (100 %). By the statistical ACORA method (Michels 2011) the behavioral data of the subfractions was correlated with the concentration and presence of the respective compounds within all subfractions to identify putative bioactive compounds that show the punishing effect.
All chemical steps of the bio-assay guided fractionation and respective chemical analysis were kindly provided by L. Wessjohann and K. Franke of the Leibniz Institute of Plant Biochemistry (IPB), Halle/Saale, Germany.

2 Animals

For all experiments, third-instar feeding-stage *D. melanogaster* larvae were used, aged five days after egg laying. All fly strains were kept in mass culture at 25° C, 60- 70 % relative humidity and at a 14 h light/ 10 h dark cycle. Cultures were maintained by daily transfering adult flies onto new food vials where they could lay eggs for 24 hours, weekly supplemented with newly emerged flies from parallel cultures. For all bio-assay guided fractionation experiments, the Canton-Special wildtype strain (CS) was used.

To study the necessity of select neurons for *R. rosea* root learning, the GAL4/ UAS method was used and crosses were carried out accordingly (see Fig. 2; Brand and Perrimon 1993). Specifically, female flies of the homozygous UAS-Kir_{GFP} [III] (expression in square brackets indicates the chromosome carrying the stated construct) effector strain (Baines et al. 2001) and male flies of the homozygous mushroom body OK107-*Gal4* [IV] driver strain (Connolly et al. 1996) were crossed, yielding double-heterozygous UAS-Kir_{GFP}/ OK107-*Gal4* animals. In these animals, a GFP-tagged Kir protein was expressed in mushroom body neurons (additional expression was reported in the larval developing optic lobe (Morante et al. 2011) as well as larval antennal lobe (Thum et al. 2011)). Because *Kir* codes for a hyperpolarizing potassium channel in the cell membrane, this prevents the mushroom body neurons from generating action potentials. In this case, the presence of the effector construct can be monitored by visualizing the GFP tag of the expressed transgene. The OK107-*Gal4* driver strain features a genomic mutation in the *white* gene on the first chromosome, which if homozygous causes the eyes of adult *D. melanogaster* to be of white color. Furthermore, the P-element driver construct for OK107-*Gal4* contained an intact mini *white* gene, restoring eyes to be 'rescued' to the wildtype, red color. Therefore, red eye color serves as a marker for the presence of the driver construct. The same principle had been used for establishing the UAS-Kir_{GFP} construct and strain. However, in this strain the genomic *white*- gene mutation was outcrossed over several generations such that the UAS-Kir_{GFP} effector strain features a wild type *white*- gene (possibly plus expression from the mini *white* on the UAS-Kir_{GFP} P-element), and thus is of red eye color. To establish the effector control, this allowed for crossing male *white* mutant flies of the w^{1118} strain (Yarali et al. 2009) to females homozygous for the UAS-Kir_{GFP} effector construct, yielding single-heterozygous UAS-Kir_{GFP}/ + flies which also are heterozygous for *white* (possibly plus expression from the mini *white* from their P-element) and thus have red eye color just as the experimental genotype. For the driver control, female flies of a CS wildtype strain were crossed with male flies of the homozygous OK107-*Gal4* strain yielding single-heterozygous +/ OK107-*Gal4* flies, again also heterozygous for *white* (possibly plus expression from the mini *white* from their P-element) and thus of red eye color. Therefore, variations in *white* gene function across experimental strains are negligible, referring to the presence of either one mini *white* (in effector and driver controls) or two mini *whites* (in the experimental genotype), in the background of genomically heterozygous *white* genes in all strains. This possibly higher gene dosage in the experimental genotype is conservative because homozygous mutations in the *white* gene, and thus a drastic loss of function, can negatively

affect associative learning between odors and punishment, at least in adult *D. melanogaster* and for electric shocks as punishment (Yarali et al. 2009).

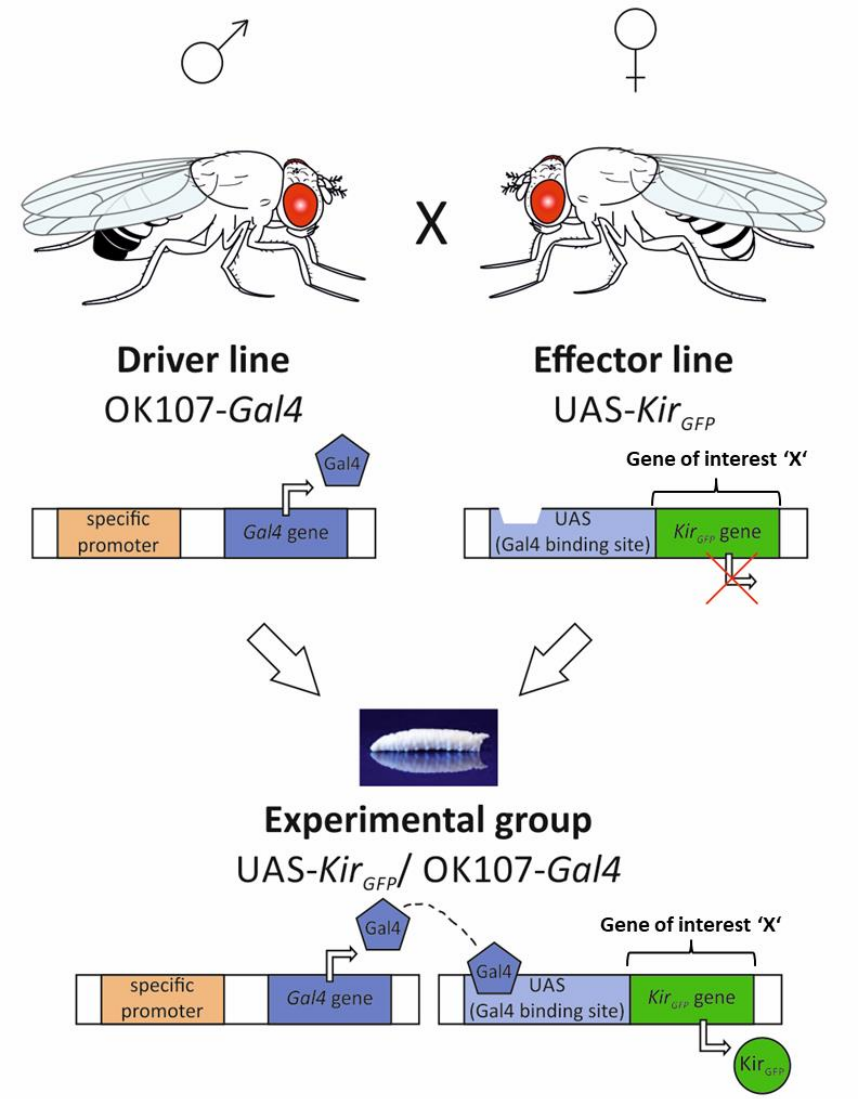

Fig. 2: Schematic overview UAS/ Gal4 system.
In this thesis, the method was used to generate *D. melanogaster* larvae lacking the ability to form action potentials in mushroom body (MB) neurons. The parental strains only carried one or the other transgenic construct in their genome, therefore not expressing the effector protein Kir_{GFP}, a GFP-tagged hyperpolarizing potassium channel. The homozygous driver strain (OK107-*Gal4*) carried the gene construct for transcription factor *Gal4* which was inserted after a specific promoter sequence in the fly's genome, enabling Gal4 expression only in MB neurons. However, as no Gal4 binding site was present in the driver line, Gal4 induced no expression of Kir_{GFP}. The homozygous effector strain (UAS-Kir_{GFP}) carried the genetic construct for Kir_{GFP} (in this case gene of interest "X") with an UAS-sequence in front of it, where transcription factor Gal4 could induce gene expression. But as in the effector strain no *Gal4* gene was present, transcription for Kir_{GFP} could not be induced. Crossing male OK107-*Gal4* with female UAS-Kir_{GFP} flies yielded heterozygous larvae (UAS-Kir_{GFP}/ OK107-*Gal4*) with both transgenic constructs. This enabled Kir_{GFP} expression in MB neurons covered by the OK107-*Gal4* expression pattern in animals of the experimental group.
Displayed *D. melanogaster* flies taken from Shimosako, N., Hadjieconomou, D., Salecker, I.: Flybow to Dissect Circuit Assembly in the Drosophila Brain. Brain Development. Vol.1082 of the series Methods in Molecular Biology. pp.57-69. (2013)
Displayed side-view of a 3D printed *D. melanogaster* larva provided with image courtesy of R. Blumenstein, Leibniz Institute of Neurobiology (LIN), Magdeburg, Germany.

3 Behavioral experiments

3.1 Odor-taste associative learning paradigm

Learning experiments followed the Pavlovian procedure introduced by Scherer et al. (2003) and Neuser et al. (2005), in a modified version as one-odor paradigm (Saumweber et al. 2011). In principle, *D. melanogaster* larvae were tested for their ability to associate an odor (conditioned stimulus, CS) with a gustatory reinforcer (unconditioned stimulus, US), followed by a test for their odor preference (conditioned response, CR).

The experiment was performed at room temperature under a fume hood. First, a cohort of 15 *D. melanogaster* larvae was taken out of their food vial and gently washed with tap water. They were carefully placed with a brush on an agarose petri dish (for manufacturer information refer to the chapter about petri dish preparation) that is supplemented with a potential taste reinforcement (+; this can be a positive reinforcer as reward or a negative reinforcer as punishment). Two Teflon odor containers (custom-made, with an inner diameter of 5 mm and perforated lids with seven 0.5 mm diameter holes) with 10 µl odor *n-amyl acetate* (diluted 1: 50 in paraffin oil; AM: CAS: 628-63-7, Merck, Darmstadt, Germany; paraffin oil CAS: 8012-95-1, Sigma-Aldrich, Seelze, Germany) were placed on opposite sides of the petri dish. The petri dish was then closed with a lid perforated at the center by about 0.5 mm diameter holes to allow aeration, and larvae could move about freely on the petri dish for 5 min before being transferred to a neutral petri dish containing pure agarose with two empty odor containers (EM) for another 5 min. This training pattern is called “paired training” because the odor stimulus and the reinforcer were presented together (AM+/ EM). This cycle was repeated two more times using fresh petri dishes each time before the larvae were tested (please note that in half of the cases, the sequence of all cycles was as indicated, and in the other half of the cases, it was reverse, i.e. EM/ AM+). For the test, the larvae were placed to the middle of a petri dish with AM and EM placed on opposite sides. To probe for appetitive memory, the test petri dish contained pure agarose, while for probing for aversive memory the reinforcer was present in the test petri dish. This was done because appetitive memory supports learned search behavior that is expressed only as long as the sought for reward is absent. On the other hand, aversive memory supports learned escape which is expressed only if the testing situation does indeed warrant escape (historically Craig 1918; Schleyer et al. 2011). After 3 min the number of larvae on the AM side of the petri dish (N_{AM}), on the EM side (N_{EM}) and in a 7 mm wide neutral zone in the middle of the petri dish ($N_{Neutral}$) were counted to calculate a preference score for the odor ($Pref_{AM}$, see equation 1) which ranges from 1 (all the animals are on the AM side) to -1 (all of the animals are on the EM side).

$$(1) \qquad Pref_{AM} = \frac{N_{AM} - N_{EM}}{N_{AM} + N_{EM} + N_{Neutral}}$$

Another group of larvae was trained reciprocally such that the odor and reinforcer were presented in an unpaired manner (AM/ EM+ or EM+/ AM) before given the choice between AM and EM in the test and calculating a preference score for the odor. Both preference scores ($Pref_{AM}$(AM+) and $Pref_{AM}$(EM+) were used to calculate a performance index (PI, see equation 2):

$$(2) \qquad PI = \frac{Pref_{AM}(AM+) - Pref_{AM}(EM+)}{2}$$

This performance index thus allowed for conclusions about the associative memory of the animals. It can reach values from 1 to -1, with positive scores indicating learned approach behavior (appetitive memory) and negative scores learned avoidance behavior (aversive memory). Zero scores suggested no learned behavior of the animals (for a schematic overview see Fig. 3).

R. rosea materials used for reinforcement were as follows:

1) 1 mg/ ml of dried *R. rosea* root material
2) 270 µg/ ml; 27 µg/ ml; 2.7 µg/ ml of *R. rosea* extract
3) 338.5 µg/ ml of water-, butanol-, ethyl acetate- and heptane fraction
4) 335 µg/ ml of ethyl acetate- and heptane subfractions

Control groups included pure agarose (1% agarose gel in *R. rosea* root experiment) or ethanol (0.1 Vol% in *R. rosea* extract and 0.17 Vol% in subfraction experiments). Experimenters were blinded to the identity and presence of the reinforcer, as well as the genotype of the tested animals.

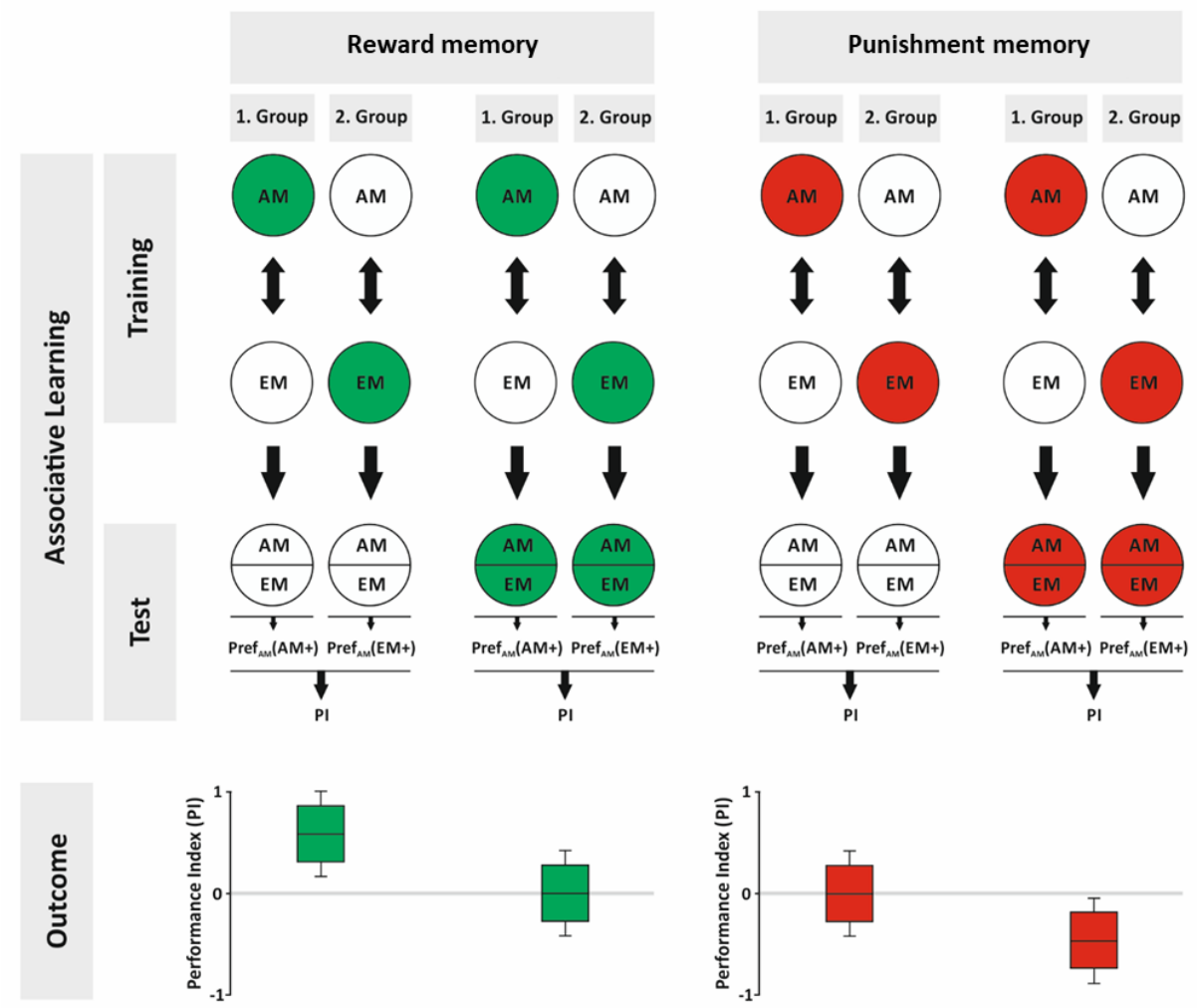

Fig. 3: Schematic overview behavioral experiment one-odor learning paradigm and expected performance indices (PI) for reward and punishment memory.
Circles indicate petri dishes containing pure 1 % agarose (white), a positive reinforcer (green) or a negative reinforcer (red). Training consisted of two reciprocal groups. Group 1 received a paired training where the odor (AM) was presented together with the reinforcer (+) for five minutes and no odor (EM) with pure agarose for five minutes (AM +/ EM). The training trials were performed three times in total. Group 2 received an unpaired training where EM, instead of AM, was presented together with the reinforcer (AM/ EM +). For the test, both groups had the choice between AM and EM for three minutes. Reciprocal preference scores for AM were calculated for the paired ($Pref_{AM}$(AM+)) and unpaired training conditions ($Pref_{AM}$(EM+)). A performance index (PI) could be calculated to quantify the learned behavior the animals showed in the test.
When a reward memory was tested in absence of the rewarding reinforcer (white), appetitive associative learning behavior was shown (positive PI values), but not when tested in presence of the reinforcer (green). When a punishment memory was tested in presence of the punishing reinforcer (red) aversive associative learning behavior was shown (negative PI values), whereas absence of the reinforcer (white) during test was insufficient to retrieve punishment memory (based on Gerber & Hendel 2006; Schleyer et al. 2011).

3.2 Naive preference tests

3.2.1 Naive odor preference

A cohort of 15 *D. melanogaster* larvae was taken out of the food vial, washed with tap water and placed on a pure agarose petri dish with an AM odor container (10 µl of *n-amyl acetate* in a 1: 50 dilution in paraffin oil) on one side and an empty container (EM) on the other side and covered with a perforated lid. After 3 min the number of animals on AM-, EM-side and a 7 mm wide neutral zone in the middle were counted and a preference score for the odor ($Pref_{AM}$ see equation 1) was calculated. Positive scores indicated a naive attraction for the odor, whereas negative scores indicated naive avoidance of the odor (see Fig. 10 for experimental setup). Please note that "naive" is used throughout to refer to animals that were not experimentally handled or stimulated in any way before the described procedures.

3.2.2 Naive *Rhodiola rosea* extract taste preference

For naive *R. rosea* taste preference tests, split petri dishes were used with *R. rosea* extract (270 µg/ ml) added to the agarose on one side and pure agarose on the other side. The larvae were placed on the petri dish covered with a perforated lid. After 3 min the number of animals on *R. rosea* extract side (RH), the 7 mm wide neutral zone (containing the border of the *R. rosea* and pure agarose phase) and the agarose side (Pure) were determined and a preference score for *R. rosea* extract ($Pref_{RH}$, see equation 3) was calculated:

$$Pref_{RH} = \frac{N_{RH} - N_{Pure}}{N_{RH} + N_{Pure} + N_{Neutral}} \quad (3)$$

Positive scores indicated a naive attraction for *R. rosea* extract, whereas negative scores meant naive avoidance (see Fig. 11 for experimental setup).

3.3 Petri dishes for behavioral experiments

Petri dishes (VWR International, Radnor, USA) with a diameter of 60 mm were prepared one day before the experiment, stored at 4° C overnight with their lids closed and upside down to prevent condensed water from dropping on the solidified agarose and brought to room temperature one hour prior to the experiment.

Pure agarose petri dishes

To produce 1 % agarose petri dishes, the amount of agarose (Carl Roth, Karlsruhe, Germany) was weighed with a scale (Kern PFB, Balingen, Germany) and added with deionized water in a glass bottle. The solution is heated up in a microwave (Samsung, Seoul, South Korea) until the

agarose is completely dissolved, poured evenly into the empty petri dish, waiting for the agarose to solidify and closing the lids.

R. rosea root petri dishes

Dried *R. rosea* root was ground for three times 30 s each at 5500 rpm (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) to produce fine powder and added to the hot and completely dissolved agarose solution to yield a *R. rosea* root concentration of 1 mg/ ml. The material was stirred on a magnetic mixer (neoLab, Heidelberg, Germany) for around 1 min to ensure a homogenous solution (same applies for the following preparations).

R. rosea extract petri dishes

For the *R. rosea* extract, three different concentrations were prepared with 100 % ethanol as solvent (280 µg/ µl; 28 µg/ µl; 2.8 µg/ µl). The volume of extract solutions being added to the hot agarose solution was the same for all dilutions (19.41 µl/ 20 ml agarose solution). This allowed for three different extract concentrations in the agarose petri dishes (270 µg/ ml; 27 µg/ ml; 2.7 µg/ ml).

R. rosea fractions petri dishes

Solutions of the *R. rosea* fractions were prepared with the solvents water (water fraction), 50% ethanol (butanol fraction), or 80% ethanol (ethyl acetate and heptane fraction). This resulted in a concentration of 135.4 µg/ µl for each fraction solution. The amount of fraction solution, which was pipetted into the hot agarose solution, was the same for all fractions (50 µl/ 20 ml agarose solution) to obtain a concentration of 338.5 µg/ ml of the respective fractions within the petri dish.

R. rosea subfractions petri dishes

Solutions of the ethyl acetate (E) and heptane (H) subfractions were all prepared with 100% ethanol: E1: 191.3 µg/ µl, E2: 197.6 µg/ µl, E3: 198 µg/ µl, E4: 193.6 µg/ µl, E5: 192.9 µg/ µl, H1: 192.8 µg/ µl, H2: 191.7 µg/ µl, H3: 192.2 µg/ µl and H4: 199.2 µg/ µl. The amount of subfraction solution to be put into the hot agarose solution differed for each subfraction ranging from 33.63 µl – 35.02 µl/ 20 ml agarose solution. This ensured a concentration of 335 µg/ ml of the respective subfraction within the petri dish.

Solvent control petri dishes

For the control groups, agarose petri dishes containing the respective solvent were prepared. The volume of solvent added to the hot agarose solution equaled the volume of the respective *R. rosea* solutions otherwise added to the agarose. For example, for the *R. rosea* extract experiment, 19.41 µl ethanol/ 20 ml agarose solution resulted in an alcoholic content of 0.1 Vol. %, whereas for the subfractions experiment 35 µl ethanol/ 20 ml agarose solution led to an alcoholic content of 0.17 Vol. % in the agarose.

Split *R. rosea* extract petri dishes

To prepare split petri dishes containing *R. rosea* extract (270 µg/ ml) on one side and pure 1 % agarose on the other side, a custom-made stamp was used as a placeholder for one half of the petri dish. First, the liquid *R. rosea* extract agarose solution (see above) was pipetted carefully into the non-stamp side of the petri dish (marked with a dot on the outside of the petri dish). After solidification the stamp was removed any excessive *R. rosea* extract agarose was removed with a scoop and the liquid pure agarose solution was pipetted into the remaining half and left for solidification.

4 Statistical analysis of behavioral data

Kruskal-Wallis tests (KW) and Mann-Whitney *U*-tests (MWU) were applied for multiple and pair-wise comparisons of the respective groups. When the same test was performed multiple times within one experiment, a Bonferroni correction was applied to keep the experiment-wide error rate at 5 %. This was done by dividing the critical *P*-value of 0.05 by the number of tests performed. Data are presented as box plots, which represent the median as the middle line, and 25 % / 75 % and 10 % / 90 % quantiles as box boundaries and whiskers, respectively. All non-parametric statistics were performed with Statistica (Statsoft, Tulsa, USA) and the one-sample sign test with R 3.4.0 (R Core Team, 2017) on a PC. Sample sizes are stated in the results. All figures were generated with CorelDRAW X6 (Corel, Ottawa, Canada) and Powerpoint (Microsoft, Redmond, USA).

5 Immunohistochemistry

To perform whole mount preparations, six larval brains of each genotype (experimental group UAS-Kir_{GFP}/OK107-*Gal4*, effector control UAS-Kir_{GFP}/+ and driver control +/OK107-*Gal4*) were dissected in ringer solution under a microscope (Zeiss, Oberkochen, Germany). Then dissected brains were fixed in 4 % paraformaldehyde dissolved in phosphate-buffered saline

(PBS) for 30 min on a shaker (Heidolph, Schwabach, Germany). After three 10 min washes in PBST (0.3 % Triton X-100; Carl Roth, Karlsruhe, Germany) on a shaker, the brains were transferred into a blocking solution, containing 5 % normal-goat-serum (NSG; Dianova, Hamburg, Germany) in PBS, for 1.5 h on a shaker. Afterward brains were incubated overnight at 4° C on a shaker with the primary antibodies, α-FASII mouse antibody to provide a structural counter staining of the brain regions of interest (diluted 1: 50 in blocking solution; DSHB, Iowa, USA) and α-GFP rabbit antibody to detect the GFP-labeled Kir_{GFP} protein (diluted 1: 1000, Invitrogen A11122, Carlsbad, USA). On the next day, six washing steps were performed with PBS for 10 min each on a shaker. After washing the brains were incubated overnight at 4° C on a shaker with the secondary antibody α-mouse Cy3 (diluted 1: 200; MoBiTec GmbH, Göttingen, Germany) and α-rabbit Alexa-488 (diluted 1: 200; Molecular Probes, Eugene, USA). After six final washing steps with PBS for 10 min each on a shaker on the next day the brains were mounted in Vectashield (Vector Laboratories Inc., Burlingame CA, USA) between two cover slips and scanned with a confocal microscope (Leica SP8, Wetzlar, Germany).

The expression pattern of all stained brains was investigated, three brains were scanned for further visualization with Fiji (Schindelin et al. 2012, open-source, maintained by the University of Wisconsin, Madison, USA) on a PC.

Results

1 Looking for a needle in a haystack: From *Rhodiola rosea* root to single bioactive compound(s)?

1.1 *Rhodiola rosea* root can be rewarding and punishing

To investigate whether *R. rosea* has reinforcing potency in larval associative learning, finely ground *R. rosea* root was used at a concentration of 1 mg/ ml and presented either paired or unpaired with an odor, followed by a test of odor preference in either case. As for the conditions for this odor preference test, the following is important: Schleyer et al. (2011) showed that larvae trained to associate a putative rewarding tastant with an odor have to be tested in absence of that rewarding tastant to behaviorally express appetitive memory. This is essential because memory-based search behavior is adaptive only as long as the sought-for reward is absent. In contrast, animals trained to associate a putative punishing tastant with an odor have to be tested in the presence of that tastant to show aversive memory. This is adaptive because a memory-directed escape from punishment is only warranted if that punishment is indeed present (also see Craig 1918 for a classical account for dove observing experiments). A schematic overview of these procedures can be found in Fig. 3.

Three experimental conditions were established. For the first two of them, training was identical, but either i) the larvae were tested in the absence of *R. rosea* root, enabling them to behaviorally express appetitive memory if such appetitive memory had indeed been formed during training. Or, ii) the larvae were tested in the presence of *R. rosea* root, thereby allowing for aversive memory to be expressed. In contrast, for the control condition iii) the animals received a mock procedure omitting *R. rosea* root during training as well as during testing. As shown in Fig. 4, memory scores across these three conditions differed (KW: $P< 0.05$; H= 27.3; df= 2; N= 42). When the larvae were tested in the absence of the root, the animals expressed appetitive memory both relative to the control condition (MWU: $P< 0.05/ 2$; U= 21; N= 15, 13) and relative to chance levels (OSS: $P< 0.05/ 3$; N= 15). When independent sets of animals were tested in the presence of the root, the animals showed aversive memory, again both relative to the control condition (MWU: $P< 0.05/ 2$; U= 31; N= 14, 13) and relative to chance levels (OSS: $P< 0.05/ 3$; N= 14). It can be concluded that *R. rosea* root has the potency to act both as a reward and as a punishment. Given that the rewarding effectors of *R. rosea* and derived materials were recently studied in detail (Michels

et al., in press), the following experiments mostly focus on the unexpected discovery of punishing effectors of *R. rosea*.

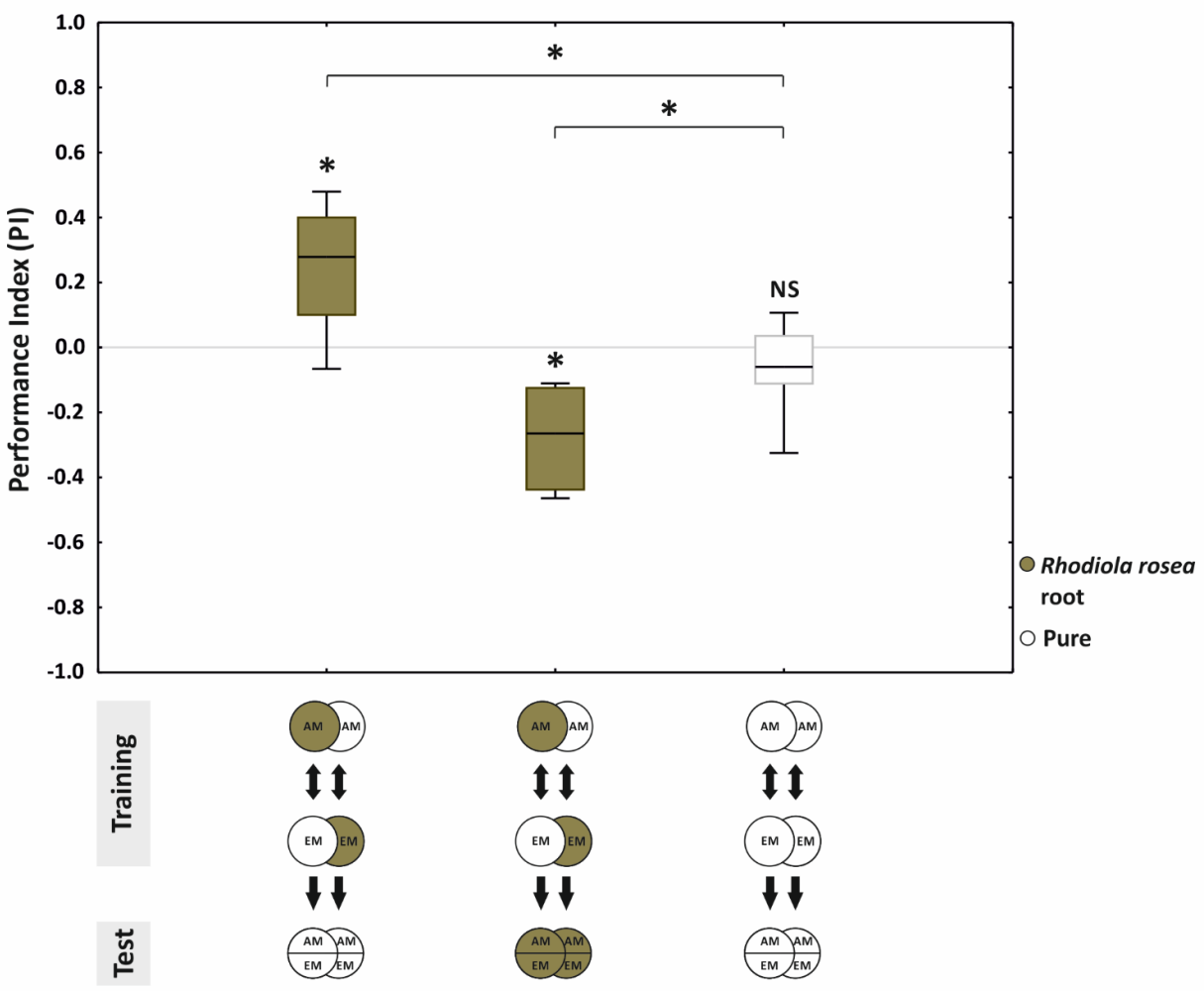

Fig. 4: *Rhodiola rosea* root can be rewarding and punishing in associative learning in WT *Drosophila melanogaster* larvae.
Experimental setup is displayed below. Circles represent agarose petri dishes, either pure (white) or supplemented with ground *R. rosea* root (brown) at a concentration of 1mg/ ml. Three experimental conditions were carried out. For two of them training was identical. *R. rosea* root was presented paired (AM) or unpaired (EM) with an odor. In the first condition, the larvae were tested in the absence of *R. rosea* root (left brown box), enabling them to behaviorally express appetitive memory. In the second condition, the larvae were tested in the presence of *R. rosea* root (right brown box), thereby allowing for aversive memory to be expressed. For the third condition (control), the animals received a mock procedure omitting *R. rosea* root during training as well as during testing (white box). Memory scores across these three conditions differed (KW: $P< 0.05$; H= 27.3; df= 2; N= 42). When the larvae were tested in the absence of the root (left brown box) the animals expressed appetitive memory both relative to the control condition (MWU: $P< 0.05/ 2$; U= 21; N= 15, 13) and relative to chance levels (OSS: $P< 0.05/ 3$; N= 15). However, when independent sets of animals were tested in the presence of the root (right brown box), the animals showed aversive memory, again both relative to the control condition (MWU: $P< 0.05/ 2$; U= 31; N= 14, 13) and relative to chance levels (OSS: $P< 0.05/ 3$; N= 14). Control condition (white box) showed no associative memory relative to chance levels (OSS: $P> 0.05/ 3$; N= 13). It can be concluded that *R. rosea* root has the potency to act both as a reward and as a punishment. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers**.** Preference scores underlying the PIs are documented in Supplementary Fig. 1.

1.2 Also *Rhodiola rosea* extract can be punishing

In the next step, an alcoholic *R. rosea* extract (provided by IBP Halle) was investigated to test whether the punishing effectors of *R. rosea* root were extractable.

As shown in Fig. 5, when using *R. rosea* extract supplemented agarose at a concentration of 270 µg/ ml an aversive memory was expressed compared to chance levels (OSS: $P< 0.05/ 4$; N= 15). With lower *R. rosea* extract concentrations (27 µg/ ml and 2.7 µg/ ml) no such aversive memory was observed (respective OSS: $P> 0.05/ 4$; N= 15, 15). As ethanol was used to dissolve the *R. rosea* extract, in the control condition animals were trained and tested with ethanol supplemented agarose (0.1 Vol %); this did not lead to an aversive memory (OSS: $P> 0.05/ 4$; N= 15). Of note, memory scores differed across the four conditions of this experiment (KW: $P< 0.05$; H= 16.8; df= 3; N= 60).

It can be concluded that the punishing effector(s) of the *R. rosea* root are extractable. For its rewarding effectors, this has been demonstrated before by Michels et al. (in press).

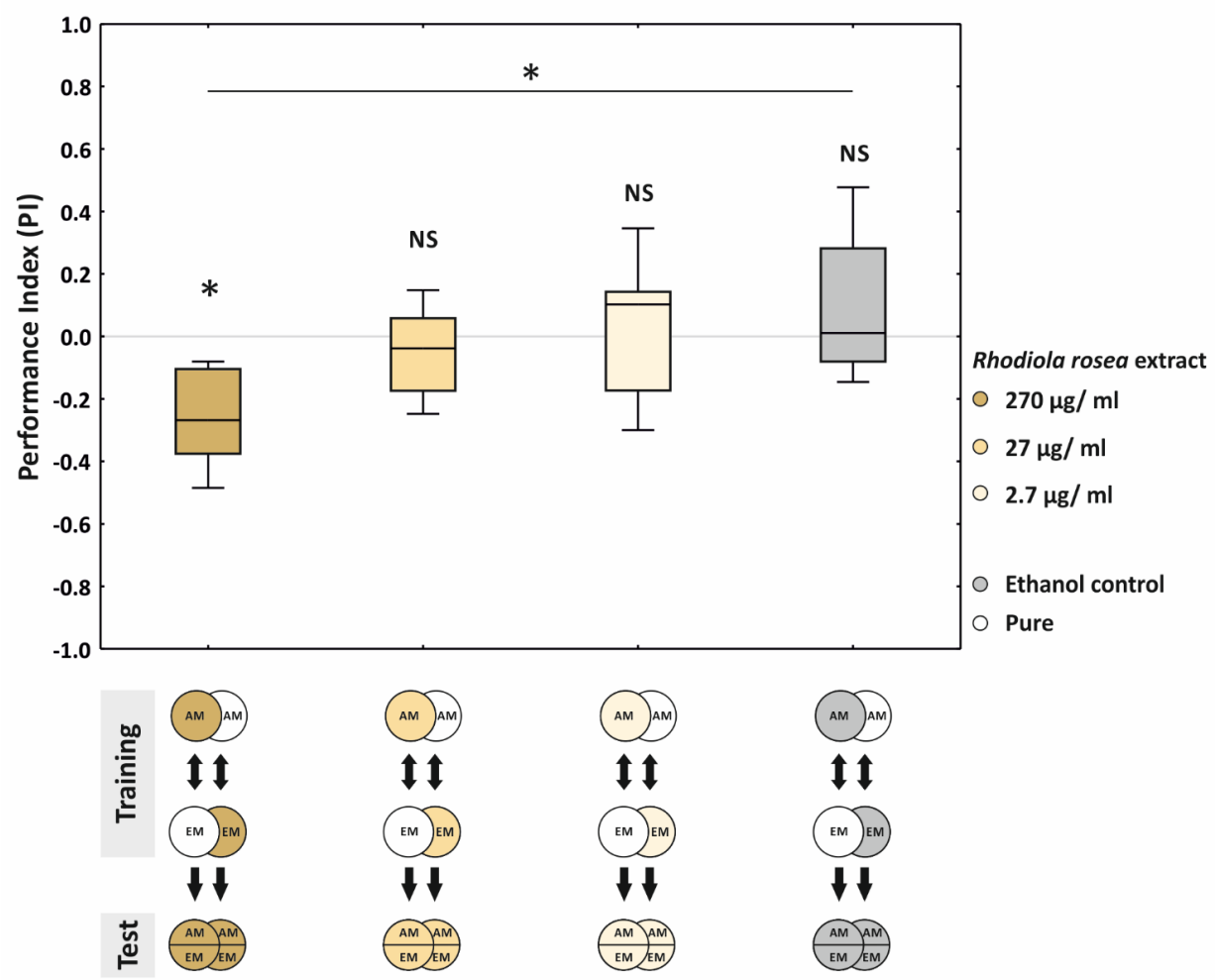

Fig. 5: *Rhodiola rosea* extract at a concentration of 270 µg/ ml can be punishing in associative learning in WT *Drosophila melanogaster* larvae but not in lower concentrations.
Experimental setup is displayed below. Circles represent agarose petri dishes, either pure (white) or supplemented with different concentrations of *R. rosea* extract (shades of gold; provided by IBP Halle) or ethanol (grey). Behavioral experiments consisted of reciprocal training groups, either extract or ethanol was presented paired (AM) of unpaired (EM) with an odor. The larvae were tested in the presence of respective extract concentration or ethanol, thereby allowing for aversive memory to be expressed The animals' odor preferences were tested, which was used to calculate a performance index (PI). When using *R. rosea* extract supplemented agarose at a concentration of 270 µg/ ml (left gold box) an aversive memory was expressed compared to chance levels (OSS: $P< 0.05/ 4$; N= 15). With lower *R. rosea* extract concentrations (27 µg/ ml and 2.7 µg/ ml; middle and right gold box) no such aversive memory was observed (respective OSS: $P> 0.05/ 4$; N= 15, 15). As ethanol was used to dissolve the *R. rosea* extract, in the control condition animals were trained and tested with ethanol supplemented agarose (0.1 Vol %; grey box); this did not lead to an aversive memory relative to chance levels (OSS: $P> 0.05/ 4$; N= 15). Memory scores differed across the four conditions of this experiment (KW: $P< 0.05$; H= 16.8; df= 3; N= 60).
It can be concluded that the punishing effector(s) of the *R. rosea* root are extractable. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers**.** Preference scores underlying the PIs are documented in Supplementary Fig. 2**.**

1.3 Ethyl acetate and heptane fractions of *Rhodiola rosea* extract can be both rewarding and punishing

Next, the compounds within the *R. rosea* extract were separated by polarity ranging from highly polar to highly nonpolar. This yielded four fractions: water, butanol, ethyl acetate and heptane fraction (provided by IPB Halle). The experiment aimed to see whether the rewarding and punishing effectors of the *R. rosea* root were still present in these four fractions, yielding eight conditions in total, given the requirement of running the test in either their presence or their absence, respectively. All *R. rosea* fractions were used at a concentration of 338.5 µg/ ml.

As displayed in Fig. 6 the testing conditions designed to uncover appetitive memory, i.e. for testing in the absence of the respective fraction, confirmed the earlier findings of Michels at al. (in press) that these fractions differed in the degree to which appetitive memory was supported (KW: $P< 0.05/ 2$; H= 45.9; df= 3; N= 99), with appetitive memory scores significantly different from chance levels for the ethyl acetate and heptane fractions (respective OSS: $P< 0.05/4$; N= 28, 27), but not water and butanol fractions (respective OSS: $P> 0.05/4$; N= 22, 22).

Also, for the testing conditions designed to uncover aversive memory, i.e. for testing in the presence of the respective fraction, memory scores differed across fractions (KW: $P< 0.05/ 2$; H= 10.9; df= 3; N= 127). Importantly for the present analysis into the effector(s) supporting aversive memory, water and butanol fractions yielded memory scores with only an aversive tendency that was, however, not significantly different from chance levels (for each fraction: OSS: $P> 0.05/ 4$; N= 31, 32). Rather, only the ethyl acetate and the heptane fractions led to an aversive memory relative to chance levels (for each fraction: OSS: $P< 0.05/ 4$; N= 32, 32).

It can be concluded that the ethyl acetate and heptane fractions, but not the water and butanol fractions, contain one or more rewarding effectors, confirming previous findings by Michels et al. (in press), and that as shown here likewise the ethyl acetate and heptane fractions, but not the water and butanol fractions, also contain one or multiple punishing effectors.

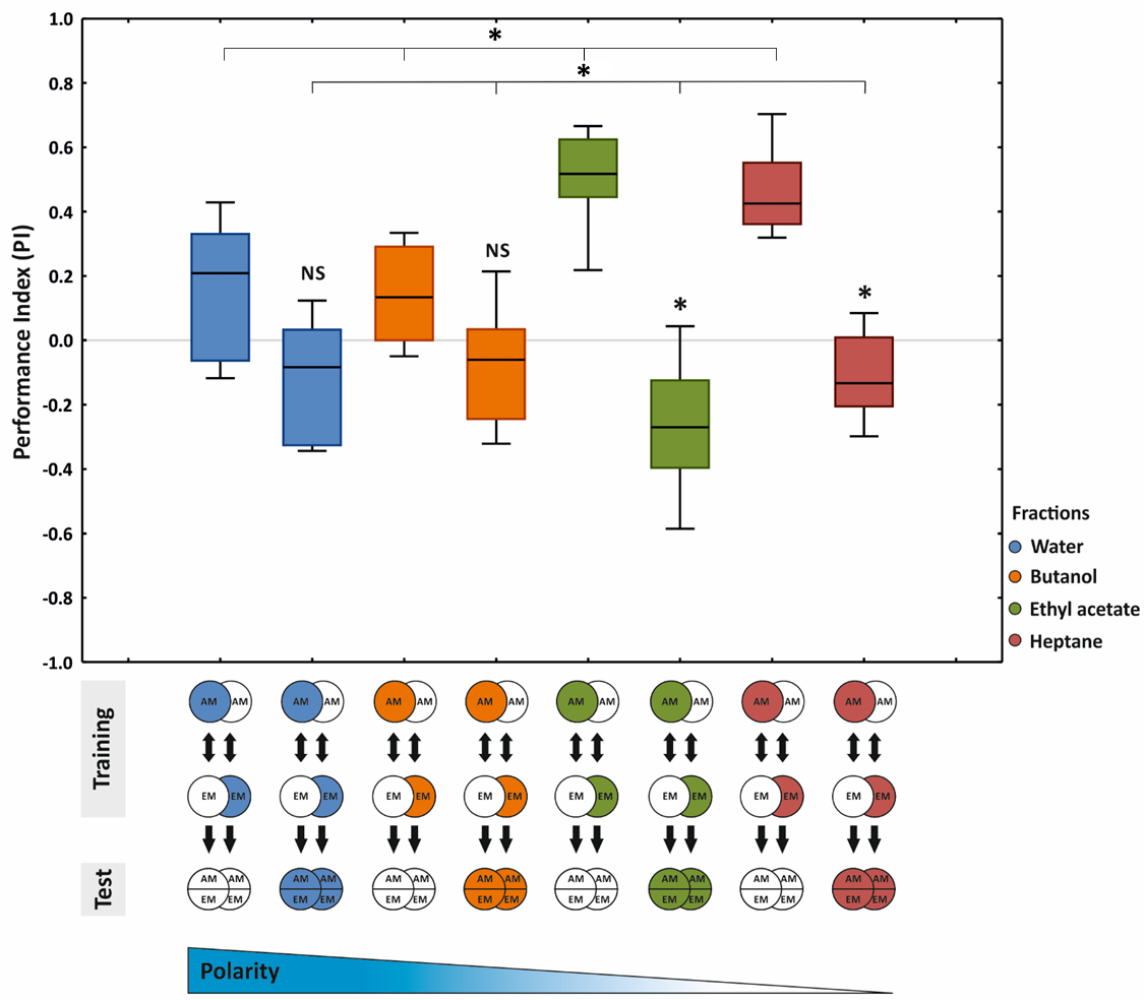

Fig. 6: *Rhodiola rosea* ethyl acetate and heptane fractions can be rewarding and punishing in associative learning in WT *Drosophila melanogaster* larvae.

Experimental setup is displayed below. Circles represent agarose petri dishes, either pure (white) or supplemented with *R. rosea* water (blue), butanol (orange), ethyl acetate (green) or heptane fraction (red). Water, butanol, ethyl acetate and heptane fraction differed in the polarity of their respective ingredients. Water fraction was containing most polar and heptane fraction most non-polar substances. The concentration of all fractions used in the experiment was 338.5 µg/ ml. Behavioral experiments consisted of reciprocal training groups, either fraction was presented paired (AM) or unpaired (EM) with an odor. Then the animals' odor preferences were tested, which was used to calculate a performance index (PI).

Total of eight testing conditions were performed to uncover appetitive memory by testing in absence of the respective fraction (left box of each fraction) and aversive memory by testing in presence of the respective fraction (right box of each fraction). Appetitive conditions confirmed the earlier findings of Michels at al. (in press) that these fractions differed in the degree to which appetitive memory was supported (KW: $P< 0.05/ 2$; H= 45.9; df= 3; N= 99).

In the aversive conditions memory scores differed across fractions (KW: $P< 0.05/ 2$; H= 10.9; df= 3; N= 127). Water and butanol fractions yielded memory scores with only an aversive tendency that was, however, not significantly different from chance levels (for each fraction: OSS: $P> 0.05/ 4$; N= 31, 32). Rather, only the ethyl acetate and the heptane fractions led to an aversive memory relative to chance levels (for each fraction: OSS: $P< 0.05/ 4$; N= 32, 32).

It can be concluded that the ethyl acetate and heptane fractions, but not the water and butanol fractions, contain one or more rewarding effectors, confirming previous findings by Michels et al. (in press), and that as shown here likewise the ethyl acetate and heptane fractions, but not the water and butanol fractions, also contain one or multiple punishing effectors. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers**.** Preference scores underlying the PIs are documented in Supplementary Fig. 3.

1.4 Ethyl acetate and heptane subfractions (E4, E5 and H1) can be punishing

To identify the punishing effector(s) from the ethyl acetate and heptane fractions, subfractions of them were produced (provided by IPB Halle). Five ethyl acetate (E1- E5) and four heptane subfractions (H1- H4) were generated with E1 being most polar and H4 most nonpolar. The subfractions were used at a concentration of 335 µg/ ml in an associative learning experiment with the respective subfraction being present during testing in order to uncover aversive memory. The control condition was performed with 0.17 % ethanol because ethanol was used as solvent of the subfractions.

As displayed in Fig. 7 memory scores differed across the 10 conditions (KW: $P< 0.05$; H= 28.8; df= 9; N= 150), with aversive memory scores of subfractions E4, E5 and H1 significantly different from control (respective MWU: $P< 0.05/ 9$; U= 14, 36, 40; N= 15, 15, 15, 15).

It can be concluded that ethyl acetate- and heptane subfractions E4, E5 and H1 contain one or more punishing effectors.

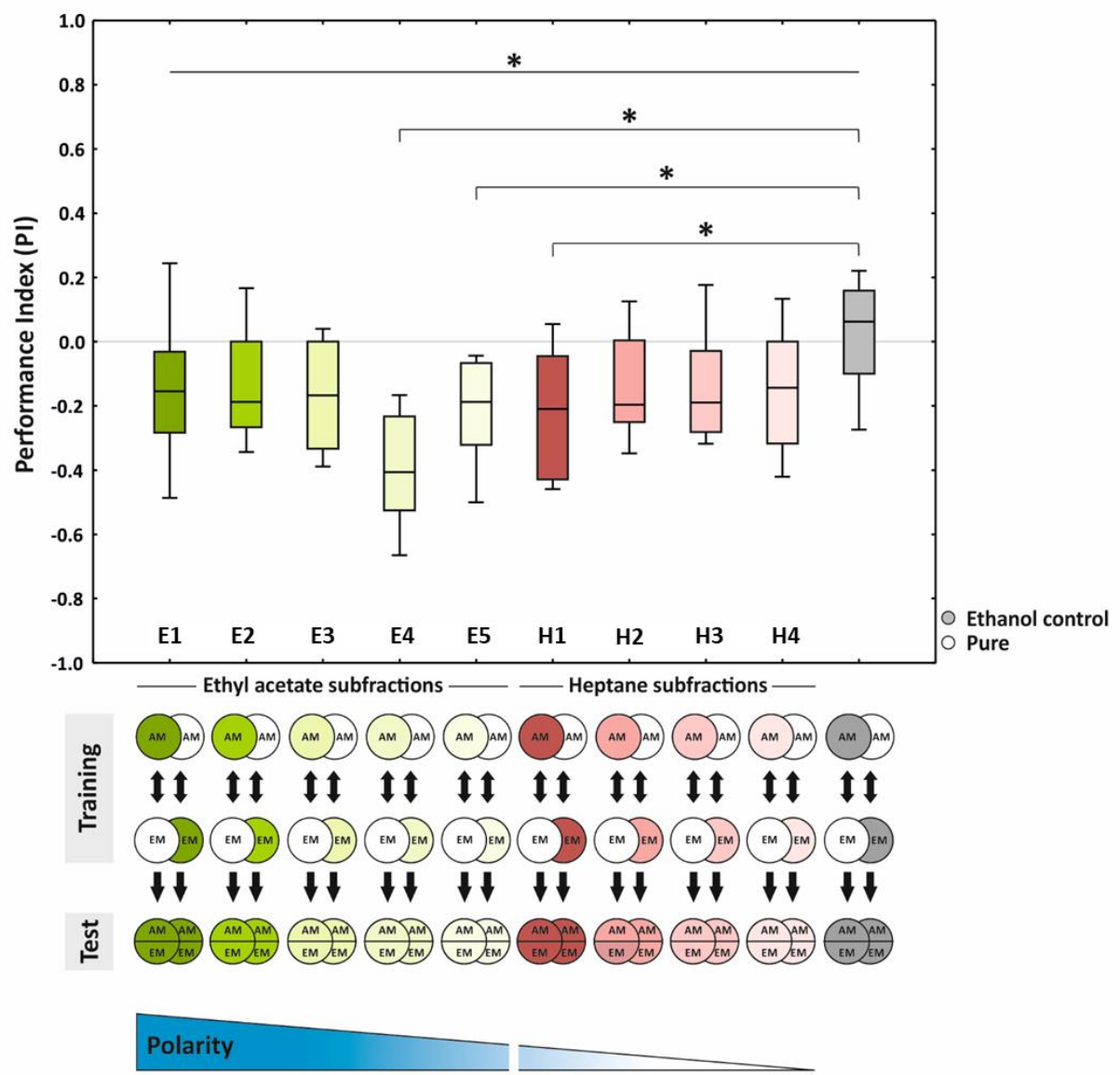

Fig. 7: Ethyl acetate and heptane subfractions (E4, E5 and H1) can be punishing in associative learning in WT *Drosophila melanogaster* larvae.

Experimental setup is displayed below. Circles represent agarose petri dishes, either pure (white) or supplemented with *R. rosea* ethyl acetate (shades of green), heptane subfractions (shades of red) with a concentration of 335 µg/ ml or ethanol (grey) at 0.17 Vol. %. Behavioral experiment consisted of reciprocal training groups, either subfraction or ethanol was presented paired (AM) or unpaired (EM) with an odor. Then the animals' odor preferences were tested, which was used to calculate a performance index (PI). All conditions were trained and tested in presence of their respective subfraction or ethanol. Five ethyl acetate- (E1-E5) and four heptane (H1-H4) subfractions were generated to identify the punishing effector(s) from the ethyl acetate and heptane fractions (see Fig. 6). The subfractions could be arranged by the polarity of their respective contained compounds, with E1 being most polar and H4 most nonpolar. Memory scores differed across the 10 conditions (KW: $P< 0.05$; H= 28.8; df= 9; N= 150), with aversive memory scores of subfractions E4, E5 and H1 significantly different from control (respective MWU: $P< 0.05/ 9$; U= 14, 36, 40; N= 15, 15, 15, 15). Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers. Preference scores underlying the PIs are documented in Supplementary Fig. 4. It can be concluded that ethyl acetate and heptane subfractions E4, E5 and H1 contain one or more punishing effectors with E4 showing the apparent strongest effect

1.5 Differences in subfraction bioactivity inadequate to identify single candidate compound(s)

After running the collected data of the behavioral experiments through the analytical ACORA method (Michels 2011) correlating the respective concentrations of the various compounds within each subfraction and fraction to the bioactive data, no candidate substance could be identified. The distribution of the behavioral data showed to be such that they could not be meaningfully compared to the chemical analysis results. Unlike for the rewarding memory scores in Michels et al. (in press), the variance of aversive memory scores between subfractions in this these was non-sufficient The so-called “hits” which were found, were interpreted as false positive by our colleagues at IBP.

2 Towards localizing necessary circuit elements for punishment learning with the *Rhodiola rosea* extract

2.1 Expression of the hyperpolarizing potassium channel Kir_{GFP} in mushroom body neurons

The mushroom bodies are a higher-order brain structure of insects and annelids, playing an essential role for the formation and behavioral expression of olfactory associative short-term memory, studied in particular detail in adult *D. melanogaster* (Heisenberg 1985; Zars 2000, Heisenberg 2003), larval *D. melanogaster* (Diegelmann et al. 2013; Gerber et al. 2009; Michels et al. 2011; Thum & Gerber 2019), and honeybees (Menzel 2001; Haenicke et al. 2018). Therefore, a mushroom body specific driver OK107-*Gal4* (Connolly et al. 1996) strain was chosen to target this set of neurons selectively. Using this driver in combination with the UAS-Kir_{GFP} effector strain allows expressing the GFP-tagged hyperpolarizing potassium channel Kir_{GFP} in mushroom body neurons, effectively preventing them from firing action potentials (Baines et al. 2001). Before doing so, the expression pattern of the GFP-tag of the Kir_{GFP} protein was tested in the brains of larvae from the experimental group, and of the genetic controls. Specifically, whole mount antibody staining for GFP and FASII, as counter staining, were performed. Only for the experimental group (UAS-Kir_{GFP}/ OK107-*Gal4*) GFP could be detected in the larval mushroom body (Fig. 8 A-C) while in the brains of both genetic controls no GFP signal was detected in the mushroom bodies (for effector control UAS-Kir_{GFP}/ + see Fig. 8 D-F; for driver control OK107-*Gal4*/ + see Fig. 8 G-I). This

confirms the expression of the Kir_{GFP} protein in the mushroom bodies of the experimental group, and its absence in the genetic controls.

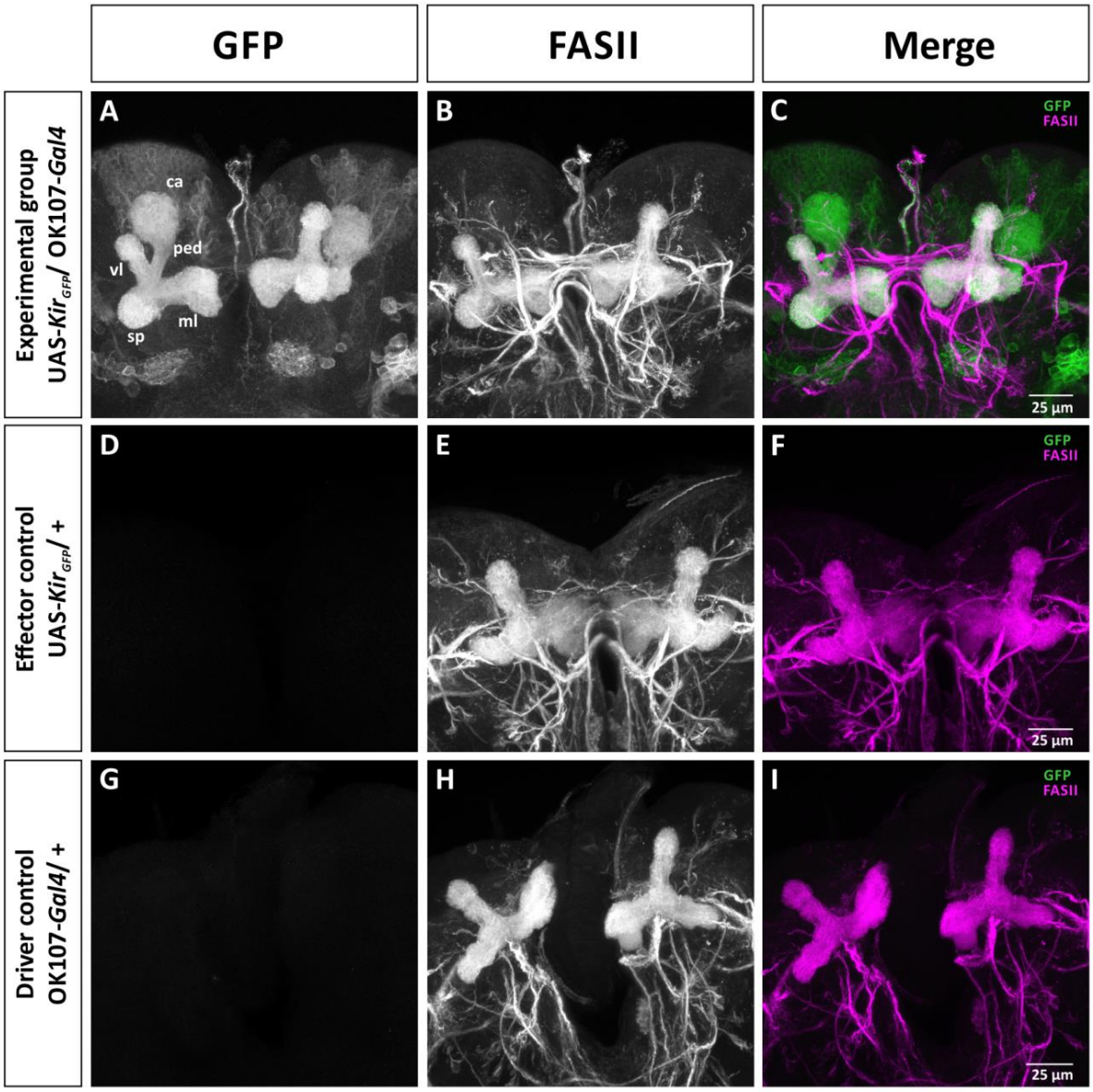

Fig. 8: Hyperpolarizing potassium channel Kir_{GFP} expression in larval mushroom body (MB) neurons of UAS-*Kir*$_{GFP}$/ OK107-*Gal4* transgenic animals but not in genetic controls.
Whole mount staining of larval brains where GFP and FASII are labeled. Rows show different genotypes and columns different stainings. In the merged pictures (third column) green represents GFP and magenta FASII staining. **A-C** experimental group (UAS-*Kir*$_{GFP}$/ OK107-*Gal4*). GFP for effector protein Kir_{GFP} and FASII for axonal background could be detected in MB neurons of larval brains. **D-F** effector control (UAS-*Kir*$_{GFP}$/ +). **G-I** driver control (OK107-*Gal4*/ +). In genetic control brains FASII but not GFP is present and detectable in larval MB neurons. For anatomical overview of larval MB labeling can be found in **A** in the left hemisphere: calyx (ca), pedunculus (ped), spur (sp), vertical lobe (vl) and medial lobe (ml). Scale bar equates 25 µm.

2.2 Silencing mushroom body neurons by Kir_{GFP} abolishes associative punishment memory scores with the *Rhodiola rosea* extract

To test if functional mushroom bodies of larval *D. melanogaster* are required for *R. rosea* punishment learning and memory, transgenic larvae with silenced mushroom body neurons (UAS-Kir_{GFP}/ OK107-*Gal4*) and the respective effector control (UAS-Kir_{GFP}/ +) and driver control (OK107-*Gal4*/ +) were used. These were trained for the association of odor with *R. rosea* extract at a concentration of 270 µg/ ml and tested for aversive memory, i.e. tested in the presence of the *R. rosea* extract.

As shown in Fig. 9 memory scores were significantly different between the genotypes (KW: $P< 0.05$; H= 9.6; df= 2; N= 42). Larvae with silenced mushroom body neurons showed memory scores significantly worse than either genetic control (UAS-Kir_{GFP}/ OK107-*Gal4* vs. UAS-Kir_{GFP}/ +; MWU: $P< 0.05/ 2$; U= 47; N= 14, 14; UAS-Kir_{GFP}/ OK107-*Gal4* vs. OK107-*Gal4*/ +; MWU: $P< 0.05/ 2$; U= 35; N= 14, 14). Of note, in these larvae memory scores were indistinguishable from chance levels (for all groups: OSS: $P> 0.05/ 3$; N= 14, 14, 14). These results give an unconclusive answer, if the establishment and/ or the behavioral expression of aversive odor- *R. rosea* extract memory requires functional mushroom body neurons.

Indeed, it was observed that the larvae in the experimental group were smaller in size than the genetic controls, suggesting developmental delay if not developmental distortion by the constitutive expression of Kir_{GFP} in the mushroom bodies. Therefore, next whether these larvae might be more generally affected in their behavior towards the odor and the *R. rosea* extract was tested.

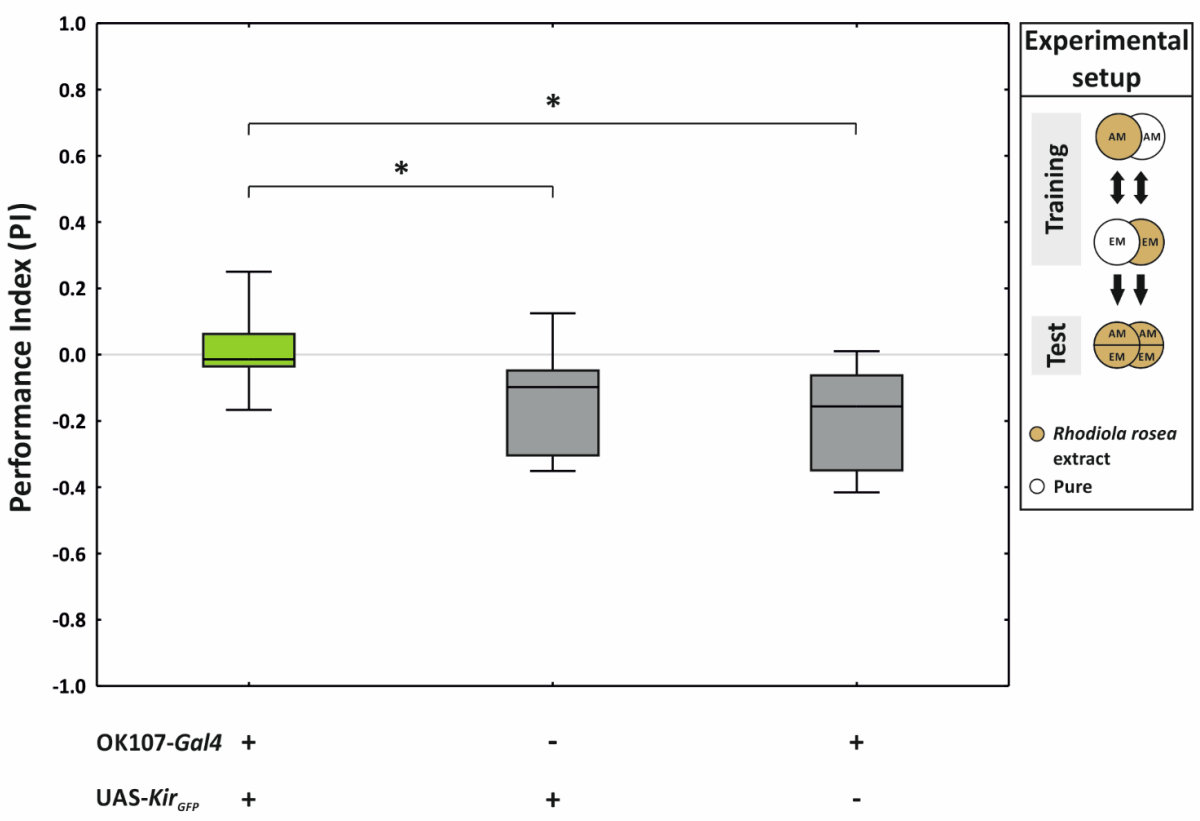

Fig. 9: Silencing mushroom body neurons by Kir_{GFP} abolishes associative punishment memory scores with the *Rhodiola rosea* extract in *Drosophila melanogaster* larvae.
Experimental setup is displayed on right side. Circles represent agarose petri dishes, either pure (white) or supplemented with *R. rosea* extract (gold) at 270 µg/ ml (effective concentration see Fig. 5). Behavioral experiment consisted of reciprocal training groups, either extract is presented with odor (AM) or without odor (EM). Then the animals' odor preferences were tested, which was used to calculate a performance index (PI). All genotypes differed from each other (KW: $P< 0.05$; H= 9.6; df= 2; N= 42) and the experimental group (green box; UAS-Kir_{GFP}/ OK107-*Gal4*) differed significantly from its genetic controls (grey boxes), both effector (UAS-Kir_{GFP}/ +; MWU: $P< 0.05$/ 2; U= 47; N= 14, 14) and driver control (OK107-*Gal4*/ +; MWU: $P< 0.05$/ 2; U= 35; N= 14, 14). Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers. Preference scores underlying the PIs are documented in Supplementary Fig. 5.

2.3 Silencing mushroom body neurons by Kir_{GFP} affects innate preference for odor and the *Rhodiola rosea* extract

As shown in Fig. 10 naive larvae of the experimental group with silenced mushroom body neurons (UAS-Kir_{GFP}/ OK107-*Gal4*) did not show a preference for the odor AM compared to chance levels (OSS: $P> 0.05$/ 3; N= 42), while the genetic controls were attracted by AM (for both controls: OSS: $P< 0.05$/ 3; N= 42, 42). Indeed, $Pref_{AM}$ differed across genotypes (KW: $P< 0.05$; H= 21; df= 2; N= 126), with $Pref_{AM}$ significantly less in the experimental group relative to the effector control UAS-Kir_{GFP}/ + (MWU: $P< 0.05$/ 2; U= 447.5; N= 42, 42) and the driver control OK107-*Gal4*/ + (MWU: $P< 0.05$/ 2; U= 431; N= 42, 42).

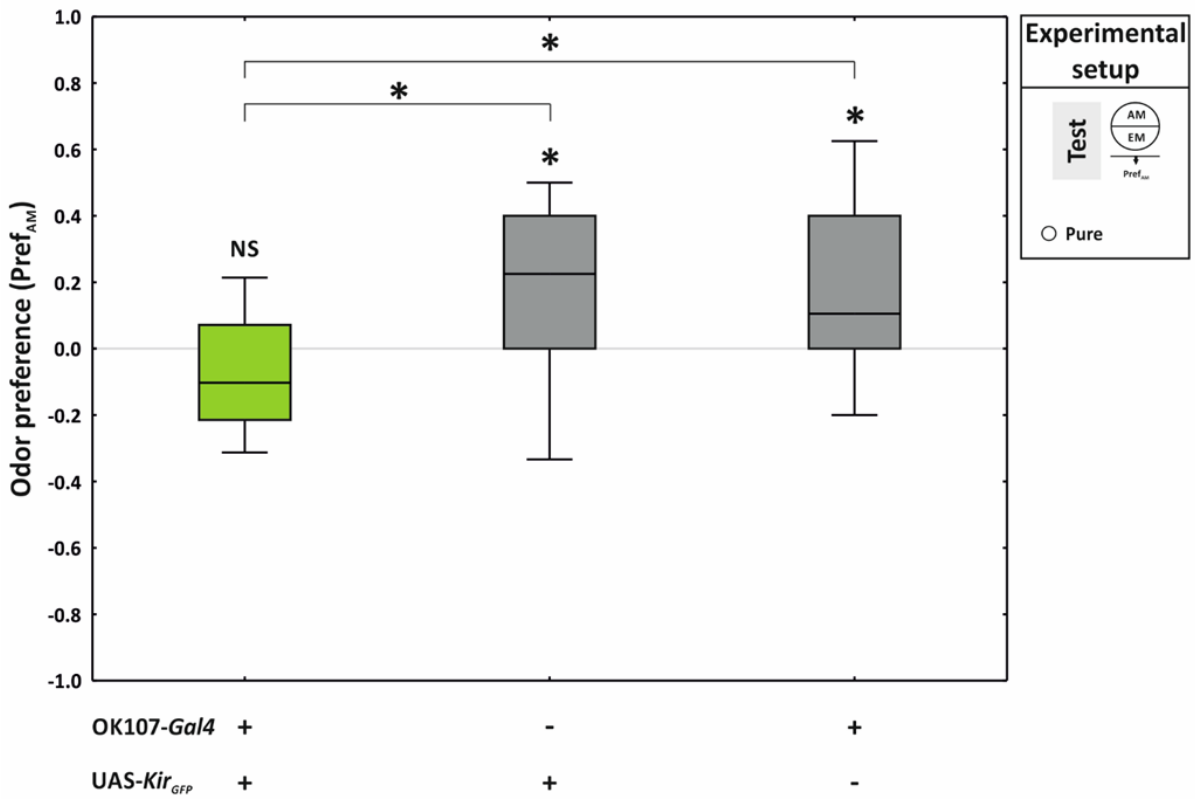

Fig. 10: Silencing mushroom body neurons by Kir$_{GFP}$ affects innate preference for odor *n-amyl acetate* (AM) in *Drosophila melanogaster* larvae.
Experimental setup is displayed on right side. Naive animals were tested for their odor preference (Pref$_{AM}$) not having had any training. The larvae had the choice between odor (AM) and no odor (EM) on a pure agarose petri dish (white circle). Experimental group with silenced mushroom body neurons (green box; UAS-*Kir$_{GFP}$*/ OK107-*Gal4*) did not show a preference for the odor AM compared to chance levels (OSS: *P*> 0.05/ 3; N= 42). Genetic controls (grey boxes; UAS-*Kir$_{GFP}$*/ + and OK107-*Gal4*/ +) were naively attracted to AM (respective OSS: *P*< 0.05/ 3; N= 42, 42). AM Preference scores differed across genotypes (KW: *P*< 0.05; H= 21; df= 2; N= 126) with AM preferences significantly less in the experimental group relative to the effector control UAS- *Kir$_{GFP}$*/ + (MWU: *P*< 0.05/ 2; U= 447.5; N= 42, 42) and the driver control OK107- *Gal4*/+ (MWU: *P*< 0.05/ 2; U= 431; N= 42, 42). An alteration in the processing of the odor AM in UAS-*Kir$_{GFP}$*/ OK107-*Gal4* larvae can be assumed. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.

Fig. 11 shows that naive larvae of the experimental group with silenced mushroom body neurons (UAS-*Kir$_{GFP}$*/ OK107-*Gal4*) showed avoidance of the *R. rosea* extract (270 µg/ ml) in a taste assay compared to chance levels (OSS: *P*< 0.05/ 3; N= 26) while both genetic controls were indifferent to the taste of the *R. rosea* extract (for both controls: OSS: *P*> 0.05/ 3; N= 26, 26). Taste preference scores indeed differed across the three genotypes (KW: *P*< 0.05; H= 17.7; df= 2; N= 78), with scores significantly more negative in the experimental group than in the effector control group UAS-*Kir$_{GFP}$*/ + (MWU: *P*< 0.05/ 2; U= 160.5; N= 26, 26) and the driver control group OK107-*Gal4*/ + (MWU: *P*< 0.05/ 2; U= 127; N= 26, 26).

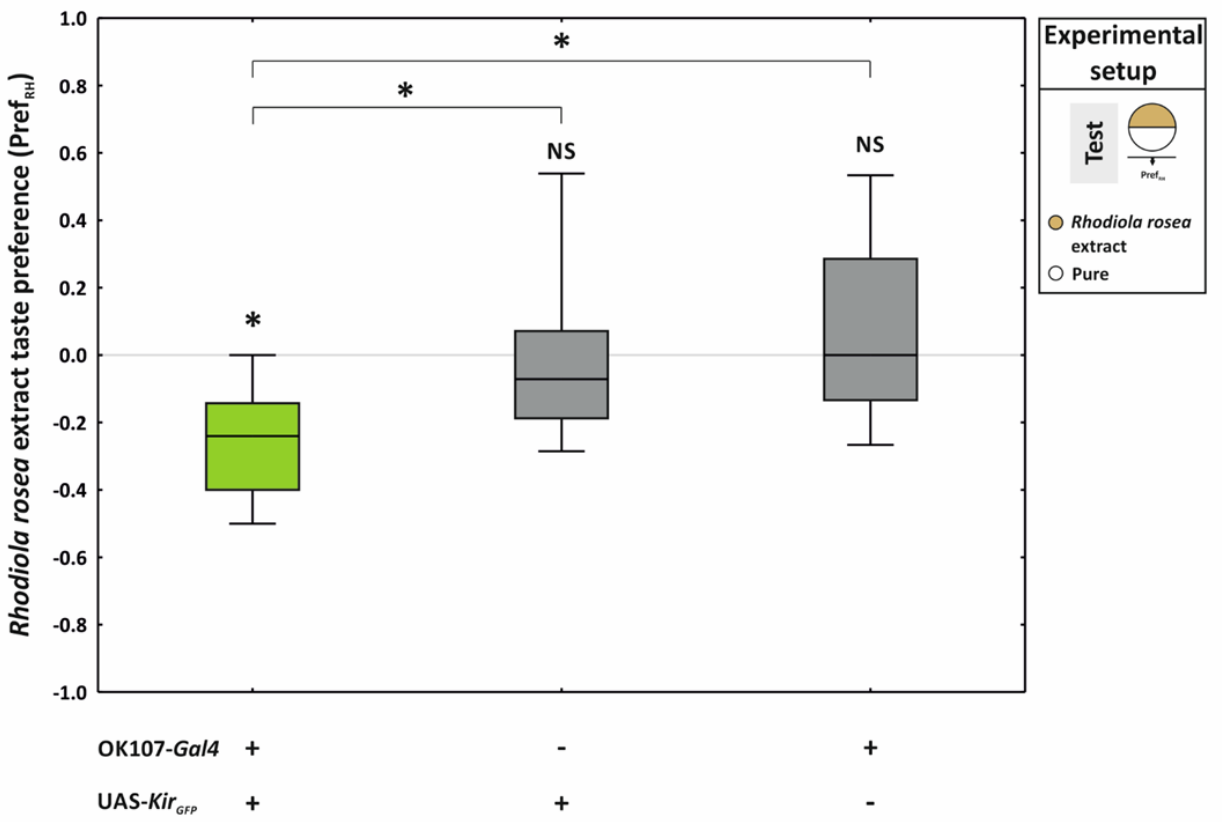

Fig. 11: Silencing mushroom body neurons by Kir_{GFP} affects innate preference for *Rhodiola rosea* extract in *Drosophila melanogaster* larvae.
Experimental setup is displayed on right side. Naive animals were tested for their naive *R. rosea* extract (270 µg/ ml) taste preference ($Pref_{RH}$) not having had any training. The larvae had the choice between pure (white) and *R. rosea* extract supplemented (gold) agarose halves of a split petri dish (circle). Experimental group with silenced mushroom body neurons (green box; UAS-Kir_{GFP}/ OK107-*Gal4*) showed naively aversion for *R. rosea* extract compared to chance levels (OSS: $P< 0.05$/ 3; N= 26). Genetic controls (grey boxes; UAS-Kir_{GFP}/ + and OK107-*Gal4*/ +) showed no preference (respective OSS: $P> 0.05$/ 3; N= 26, 26). *R. rosea* extract preference differed across genotypes (KW: $P< 0.05$; H= 17.7; df= 2; N= 78) and the experimental group differed significantly both from effector (MWU: $P< 0.05$/ 2; U= 160.5; N= 26, 26) and driver control (MWU: $P< 0.05$/ 2; U= 127; N= 26, 26). Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.

It was observed that the aversion of the *R. rosea* extract was present in the experimental group, but not in the genetic controls. This suggests that a lower speed of locomotion resulting from their smaller size cannot be the single determinant of the behavioral deficits upon silencing the mushroom body neurons in Figures 10 and 11. Otherwise, an indifference towards *R. rosea* extract due to the inability to move away in time would be expected.

It can be concluded that silencing the mushroom body neurons in the UAS-Kir_{GFP}/ OK107-*Gal4 D. melanogaster* larvae affects the behavior of experimentally naive animals towards the odor AM, and towards *R. rosea* extract in a taste assay.

Given these alterations in how the AM odor and *R. rosea* taste are processed, it must remain unresolved whether the defects in associating them reflect a genuine impairment in the formation and/ or the behavioral expression of associative memory, or whether the abolishment of memory scores as seen in Fig. 9 is secondary to more general distortions in stimulus processing as documented in Fig. 10 and Fig. 11.

Discussion

1 Conclusion from and limits of the present bio-assay guided fractionation approach

In this thesis, the applied bio-assay guided fractionation allowed to pinpoint the E4 subfraction as particularly effective as punishment (Fig. 7), at about the same strength as known tastant punishments such as quinine (Gerber et al. 2009) or salt (Niewalda et al. 2008). Therefore, it seems likely that this E4 fraction is particularly rich in punishing effectors extracted from *R. rosea*. This is remarkable because Michels et al. (in press) showed that the rewarding effectors of *R. rosea* are enriched in the E5, H1 and H2 fractions. This suggests that different compounds might be responsible for the reward and punishment effect.

However, further identification of single compound candidates by means of the ACORA method was not possible. This was likely because a multivariant data analysis such as ACORA requires actual variance across treatment conditions, which was apparently not sufficient in the present case: Performance Indices were approximately -0.4 for the E4 subfraction, but uniformly at about -0.2 for the E1-3, E5, and H1-4 subfractions (Fig. 7). In Michels et al. (in press, loc. cit. Figure 4), the rewarding effects across these subfractions were about the same in absolute strength as the punishing effects observed here but were of notably more variance. This possibly reflects that there are rewarding effectors the authors identified from *R. rosea* (namely FAE-20 and BSSG), which may distribute more broadly across the subfractions.

One way to overcome the lack of variance between the presently used subfractions in terms of their punishing effects may be to use multiple 'micro-fractions' that fall between E3-5 in polarity and repeat the present approach. Of note, even if successful, such an approach would not reveal which effector(s) are responsible for the punishing effects of the other subfractions. Given the small effect size in these cases, I would be skeptical whether these can be revealed, however.

1.1 A role for mixture interactions?

It might be argued that the bioactive substances from *R. rosea* exert their punishing effects only as a complex mixture, in the extreme case only as the full natural product (Obertreis et al. 1996; Ulrich-Merzenich et al. 2007; Yuliana et al. 2011). If so, across a bio-assay guided fractionation approach, the investigated effect would get lost with each step of extraction/ fractionation. This is, however, not what I observed: the plant material, the extract, the ethyl acetate fraction, and the E4 subfraction all have similarly strong punishing effects (Fig. 4, 5, 6, 7). Still, for the bioactive substances within the E4 subfraction, such mixture-specific effects

certainly remain possible. Of note is that mixture-specific effects, if any, did not preclude the identification of rewarding effectors from *R. rosea* (Michels et al. in press).

1.2 Alternative data analysis and screening approaches

Different methods for statistical data processing of the bioassay-guided fractionation and mass spectrometry data could be tried as the ACORA method did not lead to conclusive results with the dataset of this thesis. Alternative multivariant data analysis methods would be principal component analysis (PCA, Holmes et al. 1998; Nobeli & Thornton 2006) or hierarchical cluster analysis (HCA, Böröczky et al. 2006). Also, the ACORA method itself is 'evolving' as shown for example in the reverse metabolomics approach (Degenhardt et al. 2014, Hielscher-Michael et al. 2016). This technique is especially suitable for identifying candidate compounds from complex crude extracts. It might thus allow correlating similar but not equal *R. rosea* extracts (e.g. made from different batches of crude *R. rosea* material, as possibly different subspecies) with behavioral data. Metabolic profiles of the extracts provided by e.g. chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy would then obviate fractionation steps and their individual testing.

2 Avenues for unraveling the neuronal mechanisms of the punishing effects of *Rhodiola rosea* in *Drosophila melanogaster* larvae

The second question of the present thesis, besides the search for a punishing bio-active compound from *R. rosea*, was to identify neurons required for this punishing effect. Using the *R. rosea* extract and transgenic larvae permanently preventing the neurons of the mushroom body from firing action potentials (UAS-Kir_{GFP}/ OK107-*Gal4*) did not yield conclusive results, however. Although punishment memory scores for the association of odor with *R. rosea* extract were impaired in the experimental genotype compared to the genetic controls (Fig. 9), behavioral control experiments for naive odor and taste responses also showed differences between experimental and control genotypes (Fig. 10, 11). In particular, the absence of naive odor preference in the experimental genotype allows arguing that the larvae might be unable to detect the odor and were therefore handicapped to associate it with the punishment conferred by the *R. rosea* extract. Of note, in the naive taste preference test the experimental genotype showed aversion to the *R. rosea* extract than the genetic controls, indicating that the experimental genotype was able to taste the extract, and that locomotor abilities or speed were not generally impaired, despite the smaller size of the experimental genotype. Given this lack of behavioral specificity in the effect of silencing the mushroom body neurons, it remains

unclear whether these neurons are involved in not only odor preference and preference for the *R. rosea* extract, but also in the association process of odor and the punishment by that extract. In the following, I want to discuss how this could be resolved.

2.1 Using a different effector

While keeping the OK107-*Gal4* expression pattern, using the effector strain UAS-*shibire*^{ts1} (Kitamoto 2001), which blocks chemical-synapse output in a temperature-dependent manner only during the experiment, would allow for a physiological neuron functioning during larval development in order to eliminate possible developmental deficits in UAS-*Kir*$_{GFP}$/OK107-*Gal4*. However, the temperature switch from room temperature to 30°C that is required for such experiments can also affect naive behavioral responses (Honjo & Furukubo-Tokunaga 2009; Pauls et al. 2015).

Using UAS-*TnTe* (Sweeney et al. 1995) would produce the tetanus toxin light chain in the neurons covered by OK107-*Gal4* and would prevent synaptic output by disrupting synaptobrevin function, thus preventing transmitter vesicle fusion. In combination with the temperature-sensitive inhibitor of *Gal4*, *Gal80*^{ts}, this technique could be used in an acute manner, largely preventing developmental defects (Kamikouchi et al. 2009; Jones 2009).

Of note, despite the advantage of being relatively acute in effect, both these procedures leave action potential firing as well as the function of electrical synapses intact and are thus less comprehensive in effect than silencing these neurons altogether.

A further possibility would be to combine *Gal80*^{ts} and UAS-*ΔOrk* (Nitabach et al. 2002), which codes for an inward rectifying potassium channel and thereby, like UAS-*Kir*$_{GFP}$, inhibits cell depolarization. However, according to Pauls et al. (2015) this effector is less potent than Kir$_{GFP}$.

In all these cases, a direct observation of transgene expression is problematic, however, because the transgenes are not GFP-tagged or because suitable antibodies are not available. Therefore, a reasonable next step would be to maintain UAS-*Kir*$_{GFP}$ and a suitable *Gal4* driver (see next section) and to combine it with *Gal80*^{ts}.

2.2 Using a different driver

2.2.1 MB driver strain

If it turned out that even using UAS-*Kir*$_{GFP}$/OK107-*Gal4* in combination with *Gal80*^{ts} still leads to behaviorally unspecific effects on the association between odor and the punishment conferred by *R. rosea* extract, a different mushroom body driver strain could be used that

shows expression in a less broad set of mushroom body neurons as compared to OK107-*Gal4*. Honjo & Furukubo-Tokunaga (2009) demonstrated synaptic output of larval MB neurons covered by driver lines 201y and OK301 (Connolly et al. 1996) is essential for retrieval but not acquisition of quinine memory, as it is the case in adult flies (Dubnau et al. 2001). 201Y shows a rather broad expression pattern also outside of the MB (Pauls et al. 2010), whereas OK301 represents a more restricted subset (Honjo & Furukubo-Tokunaga 2005). Therefore, choosing a driver lines to cross with UAS-Kir_{GFP} has to be considered with caution as manipulation in a broad spectrum of neurons might lead to unwanted phenotypes.

2.2.2 Involvement of dopaminergic mushroom body input neurons?

Given that the mushroom body neurons have been shown to be involved in all olfactory associative memories studied for such involvement in adult and larval *Drosophila melanogaster* to date (Heisenberg 1985; Heisenberg 2003; Guven-Ozkan & Davis 2014; Widmann et al. 2018; Thum & Gerber 2019), it seems rather likely that this will turn out to be the case for the association of odor with punishment by *R. rosea* extract as well. As the olfactory input to the mushroom body is known to be carried by olfactory projection neurons (Vosshall & Stocker 2007), one naturally wonders which mushroom body input neurons mediate the punishing effect of the *R. rosea* extract. Indeed, it is known that dopaminergic mushroom body input neurons are essential for the acquisition of a punishment memory (Schwaerzel et al. 2003). Especially the interferences of dopamine-neurons covered by the TH-*Gal4* driver line (Friggi-Grelin et al. 2003) which uses the promoter sequence for tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis, led to an impairment of punishment learning in adult flies (Schwaerzel et al. 2003; Yarali & Gerber 2010) as well as in larval *D. melanogaster* (Selcho et al. 2009). Activating TH-*Gal4* neurons can confer a punishing effect (Schroll et al. 2006). Specifically, dopaminergic projections to the mushroom body are essential for the acquisition, but not for the retrieval of aversive memory (Schwaerzel et al. 2003). TH-*Gal4* is a broad driver with an expression pattern in more than 75% of dopaminergic neurons in the larval brain (Selcho et al. 2009) and could thus be used in a first step to test for the requirement of dopaminergic neurons in the present paradigm. In a next step, more specific drivers could be used, covering any one of the three dopaminergic neurons recently discovered to mediate punishment (Eschbach et al. 2020), and possibly disentangling the role of different dopamine receptors in this process. Impairments in aversive and appetitive odor-taste learning have been found for *dumb* mutants in larvae (Selcho et al.

2009). Of note, the rewarding effects of the *R. rosea* extract appear to be independent of the known dopaminergic pathways for reward processing Michels et al. (in press).

Summary

Rhodiola rosea is a plant native to the arctic regions and used in traditional medicine in humans. The aim of this thesis is to investigate the reinforcing effects that *R. rosea* has in larval *D. melanogaster*, an established study case for associative learning. *R. rosea* root material was previously known to have a rewarding effect in a one-odor learning paradigm in *D. melanogaster* larvae. Here it is shown that, in addition, this plant material has as punishing effect. Given that a compound that can mediate the rewarding effects of *R. rosea* has recently been identified (Michels et al. in press), it was the aim of this thesis to identify the chemical compound(s) responsible for the punishing effect. Towards this end a bio-assay guided fractionation with activity-correlation analysis (ACORA) was performed. Each fractionation step was tested in the one-odor learning paradigm for effectiveness. In the end a single compound could not be identified. The results suggest, however, that the punishing and the rewarding effects of *R. rosea* are not mediated by the very same compound.

The second part of this thesis focuses on the neuronal circuitry underlying the punishing effect of *R. rosea* in *D. melanogaster* larvae. Transgenic manipulation of the larval mushroom body, a brain area known for memory formation, was performed using the UAS/ Gal4 system. UAS-Kir_{GFP}/ OK107-*Gal4* animals showed impairments in *R. rosea* extract punishment memory. However, experiments for innate responses towards odor and *R. rosea* extract showed differences to genetic control animals, such that these differences might partially account for the observed punishment memory defects.

In conclusion, as the ACORA method did not result in a candidate substance, alternative approaches would be needed to possibly identify the compound(s) mediating the punishing effect of *R. rosea*. Also, in order to investigate the neuronal mechanism of *R. rosea* punishment memory in larval *D. melanogaster*, different transgenic animals without unwanted effects to naive behavior would be required.

Zusammenfassung

Diese Arbeit beschäftigt sich mit Grundlagenforschung für neue pharmakologische Ansätze hinsichtlich der belohnenden und bestrafenden neuronalen Systeme. Die Wurzel der Pflanze *Rhodiola rosea* findet in der Naturheilkunde beim Menschen Anwendung. Diese Arbeit untersucht eine akute motivationale Wirkung von *R. rosea* als Strafreiz beim assoziativen Lernen. Experimente mit *Drosophila melanogaster* eignen sich zur Erforschung dieser Prozesse, da sie sich als Modelle etablieren konnten, um wichtige Rückschlüsse auf basale Vorgänge beim Lernen ziehen zu können. In Verhaltensexperimenten des „Duft-Lernparadigma“ konnte sowohl ein zuvor bereits bekannter belohnender als auch, überraschender Weise, ein bisher nicht bekannter bestrafender Einfluss von *R. rosea*-Wurzelmaterial nachgewiesen werden. Um herauszufinden, welche Substanz(en) für den bestrafenden Effekt verantwortlich sind, wurde in Zusammenarbeit mit dem IPB Halle eine „bio-assay guided fractionation“ mit Aktivitäts-Korrelations-Analyse (ACORA) von *R. rosea* durchgeführt. Die einzelnen Schritte wurden auf ihr Wirksamkeit im Verhaltensexperiment geprüft. Dieser Arbeit gelang die Identifikation einer bestrafenden Kandidatensubstanz jedoch nicht, obwohl die Zwischenschritte signifikante Wirksamkeit zeigten. Hingegen gelang in Michels et al. (im Druck) die Identifizierung einer belohnenden Substanz aus *R. rosea* mit Hilfe dieser Methodik. Die Ergebnisse der vorliegenden Arbeit deuten aber darauf hin, dass andere Substanzen für den bestrafenden Effekt verantwortlich sind.

Ein weiterer Aspekt der Arbeit beschäftigte sich mit dem neuronalen Wirkmechanismus des *R. rosea* Straflernens. Dazu wurde das UAS/ Gal4 System angewendet um mit Hilfe der Expression von Transgenen Nervenzellen des Pilzkörpers, einer wichtigen Schaltstelle der Gedächtnisbildung im Larvengehirn, zu manipulieren. Tatsächlich zeigten diese Tiere im Bestrafungslernen mit *R. rosea* eine Schwäche, allerdings ergaben Kontrollexperimente auch Beeinträchtigungen naiven, angeborenen Verhaltens durch die Transgenexpression. Zusammenfassend ist weiterführende Forschung beispielsweise mit Alternativen zu ACORA und die Verwendung anderer, mit potentiell weniger Nebenwirkungen behafteter Transgene mittels des UAS/ Gal4- Systems nötig um die Fragen dieser Arbeit hinreichend beantworten zu können.

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Ehrenerklärung (Declaration)

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

„Punishing Effect of *Rhodiola rosea* in larval *Drososphila melanogaster*“

im Leibniz Institut für Neurobiologie, sowie Institut für Pharmakologie und Toxikologie der Medizinischen Fakultät der Otto-von-Guericke-Universität Magdeburg

mit Unterstützung durch das Leibniz Institut für Pflanzenbiochemie, Halle (Saale)

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 20.07.2020

Unterschrift

Erklärung zur strafrechtlichen Verurteilung

Ich erkläre hiermit, nicht wegen einer Straftat verurteilt worden zu sein, die Wissenschaftsbezug hat.

Magdeburg, den 20.07.2020

Unterschrift

Darstellung des Bildungsweges (Curriculum vitae)

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Magdeburg, den 20.07.2020

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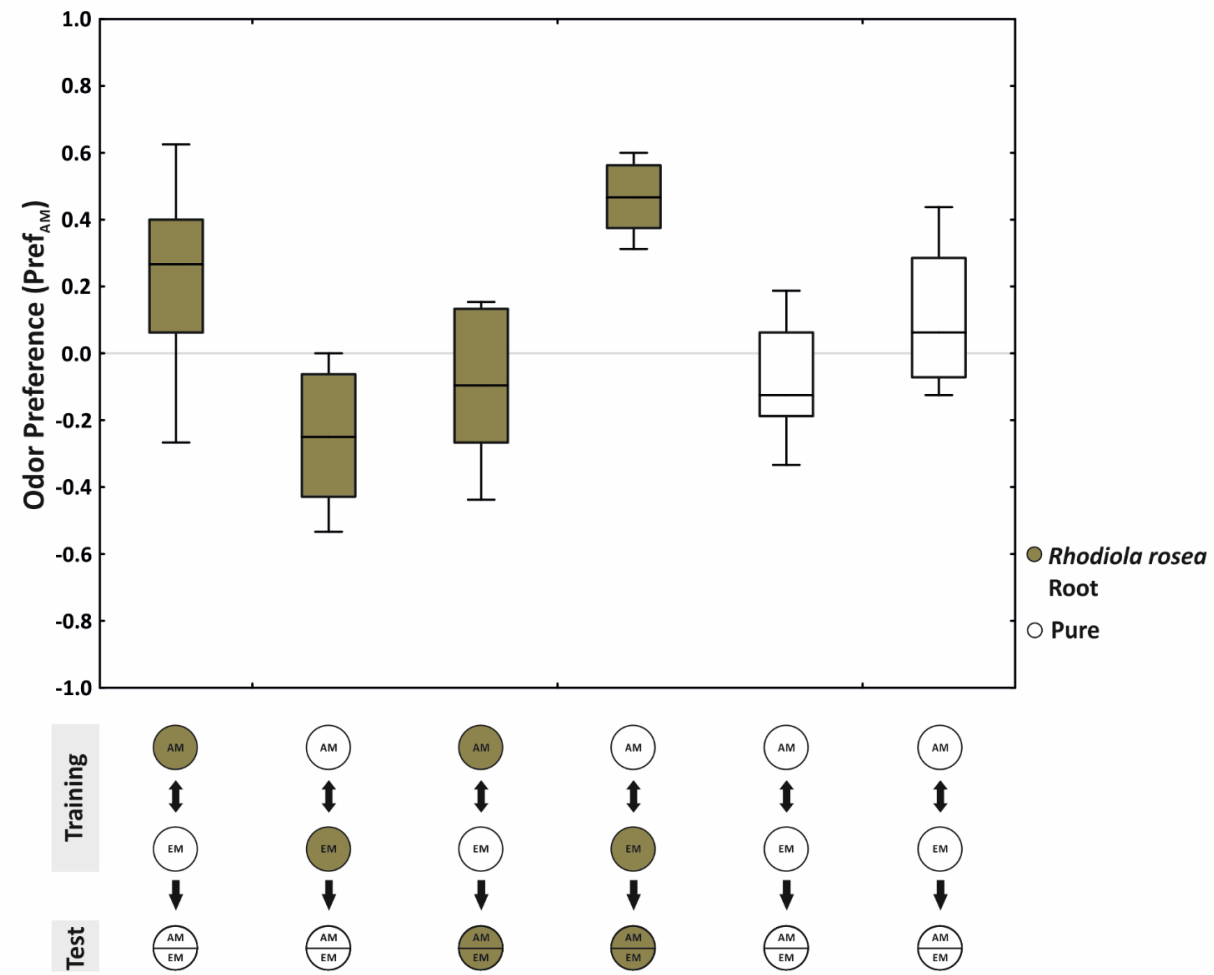
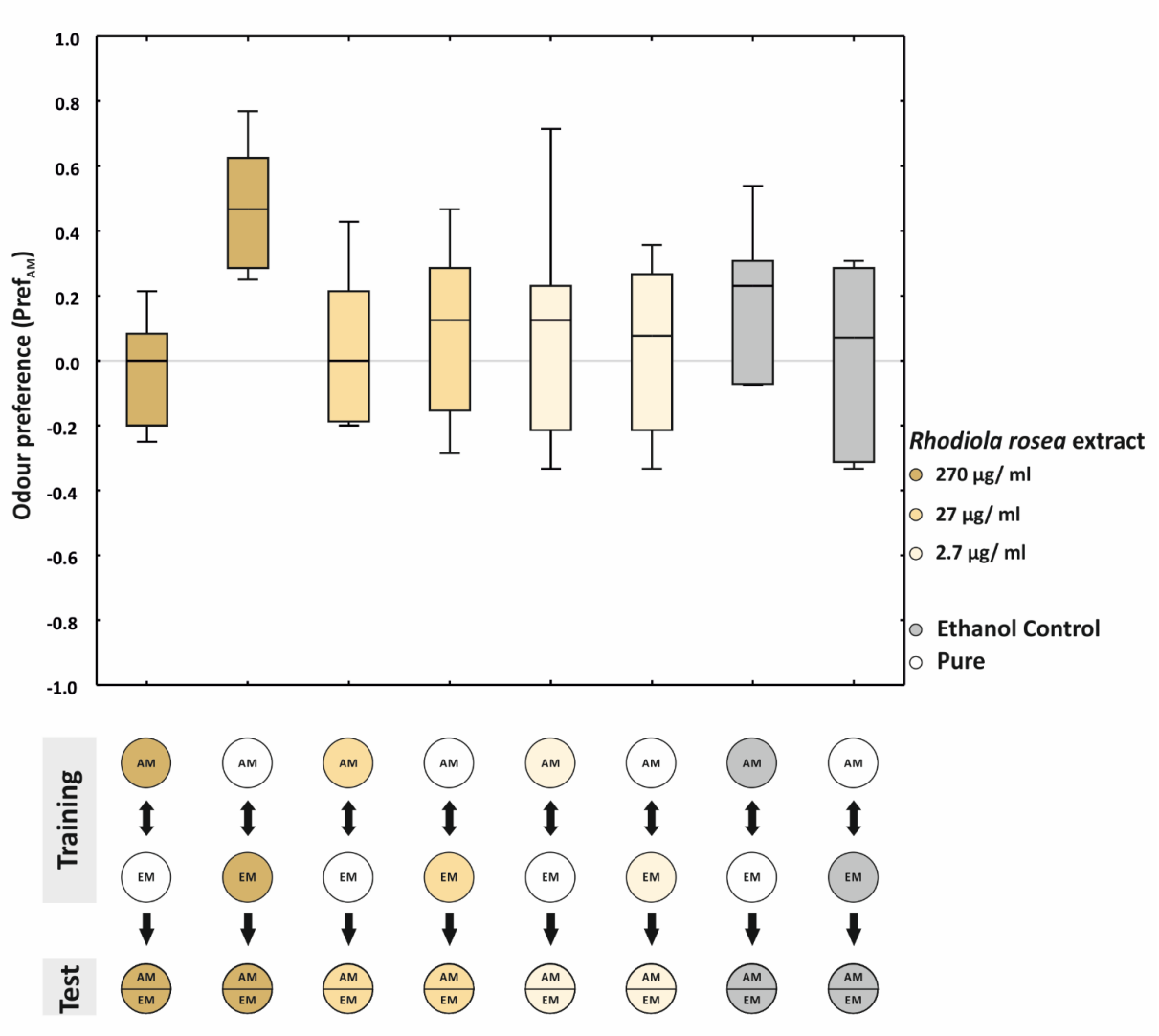
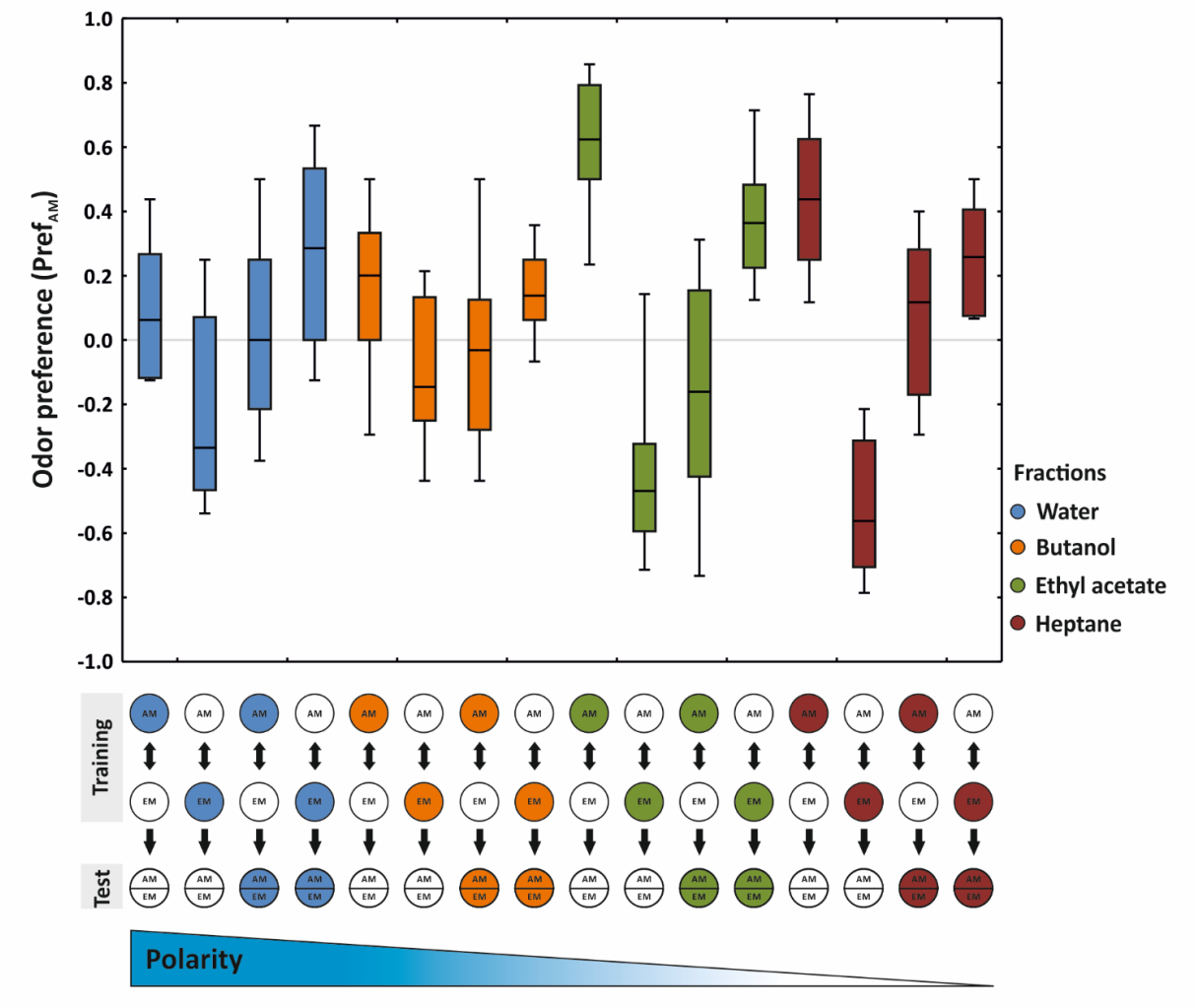
Supplementary Material



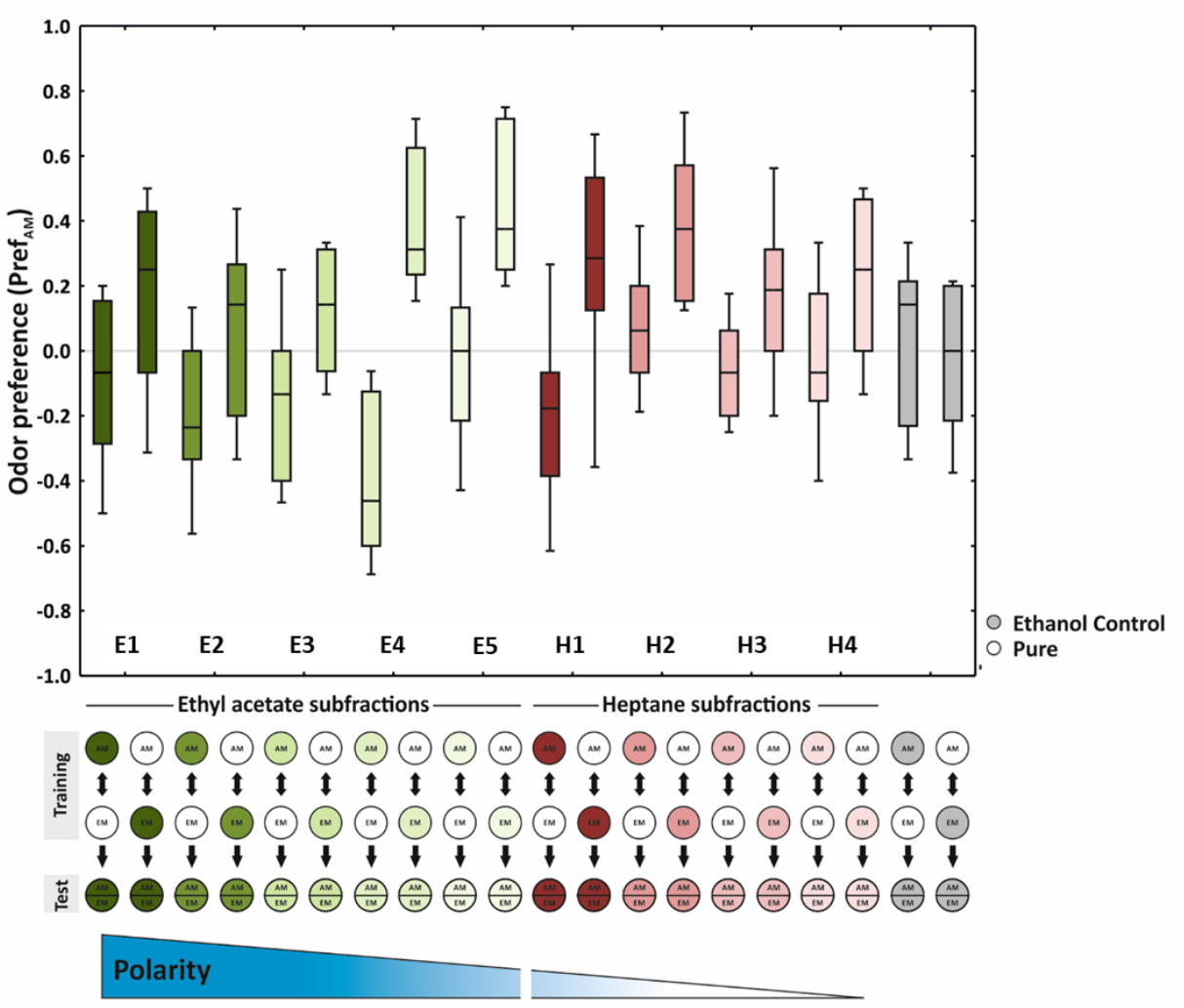
Supplementary Fig. 1: Preference scores underlying the Performances Indices from Fig. 4. Documentation of the *n-amyl acetate* (AM) preference scores ($Pref_{AM}$) underlying the Performance Indices from Fig. 4. Three groups are shown (from left to right: *R. rosea* root in rewarding setup (N= 15), *R. rosea* root in punishing setup (N= 14) and control group (N= 13)). Preference scores after paired (respective first box plot) and unpaired (respective second box plot) training are shown separately for each group. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.



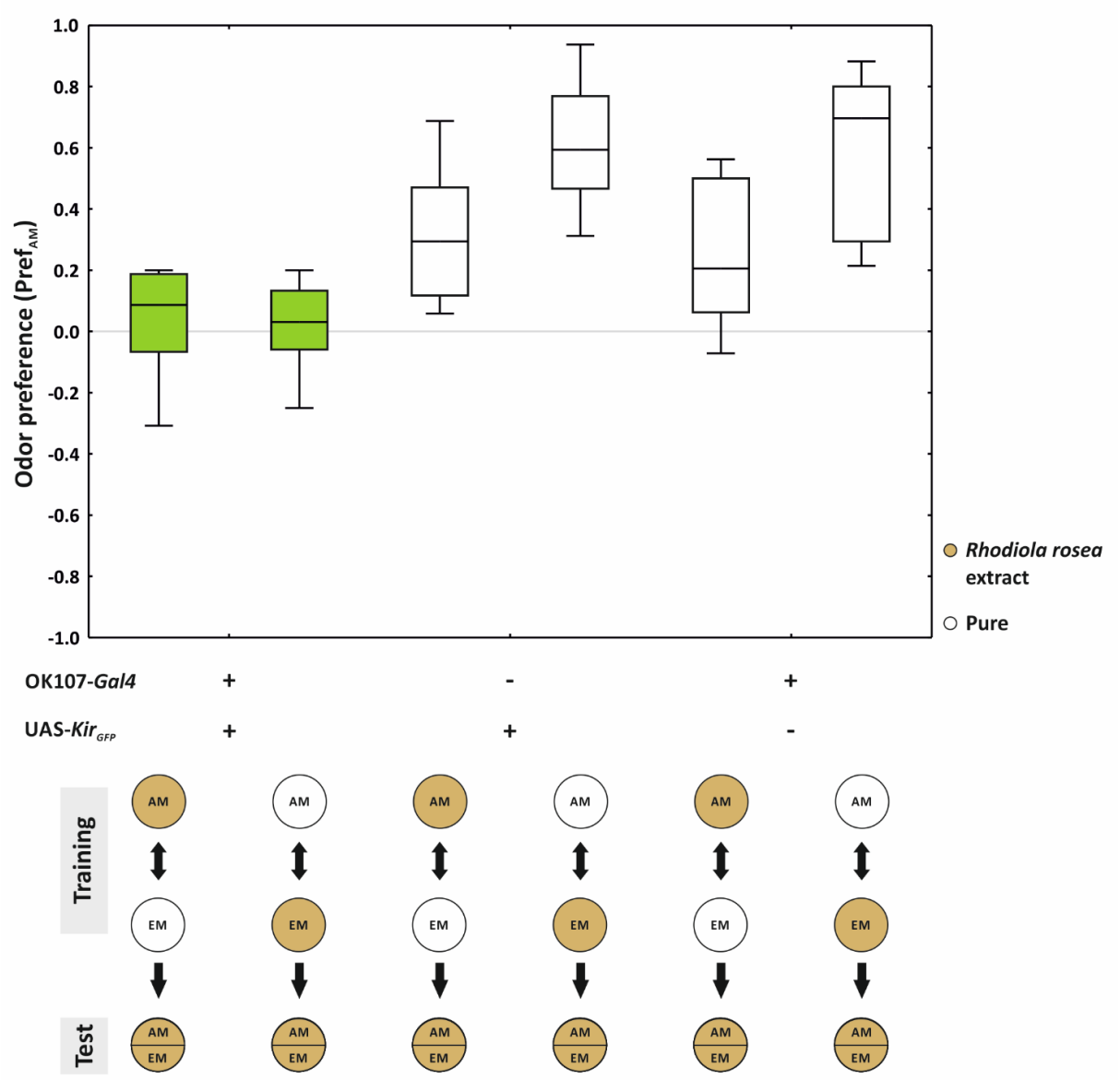
Supplementary Fig. 2: Preference scores underlying the Performances Indices from Fig. 5. Documentation of the *n-amyl acetate* (AM) preference scores ($Pref_{AM}$) underlying the Performance Indices from Fig. 5. Four groups are shown (from left to right: *R. rosea* extract at different concentrations (270 µg/ ml, 27 µg/ ml, 2.7 µg/ ml) and control group, respective N= 15). Preference scores after paired (respective first box plot) and unpaired (respective second box plot) training are shown separately for each group. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.



Supplementary Fig. 3: Preference scores underlying the Performances Indices from Fig. 6. Documentation of the *n-amyl acetate* (AM) perference scores ($Pref_{AM}$) underlying the Performance Indices from Fig. 6. Eight groups are shown (from left to right: water fraction in rewarding (N= 22) and punishing (N=32) setup, butanol fraction in rewarding (N= 22) and punishing (N=32) setup, ethyl acetate fraction in rewarding (N= 28) and punishing (N=32) setup, heptane fraction in rewarding (N= 27) and punishing (N= 32) setup). Preference scores after paired (respective first box plot) and unpaired (respective second box plot) trainings are shown separately for each group. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.



Supplementary Fig. 4: Preference scores underlying the Performances Indices from Fig. 7. Documentation of the *n-amyl acetate* (AM) preference scores ($Pref_{AM}$) underlying the Performance Indices from Fig. 7. Ten groups are shown (from left to right: five ethyl acetate E1-E5 and four heptane H1-H4 subfractions with decreasing polarity and control group, respective N= 15). Preference scores after paired (respective first box plot) and unpaired (respective second box plot) training are shown separately for each group. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.



Supplementary Fig. 5: Preference scores underlying the Performances Indices from Fig. 9. Documentation of the *n-amyl acetate* (AM) preference scores ($Pref_{AM}$) underlying the Performance Indices from Fig. 9. Three groups are shown (from left to right: experimental group UAS-Kir_{GFP}/ OK107-*Gal4*, effector control UAS-Kir_{GFP}/ + and driver control OK107-*Gal4*/ +, respective N= 14). Preference scores after paired (respective first box plot) and unpaired (respective second box plot) training are shown seperately for each group. Box plots show median as middle line, 25 % and 75 % quantiles as box boundaries and 10 % and 90 % quantiles as whiskers.