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Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth Heliothis virescens

Contents

Papers included in the thesis

I. Jørgensen K., Kvello P., Almaas T.J., Mustaparta H. (2006). Two closely located areas in the suboesophageal ganglion and the tritocerebrum receive projections of gustatory receptor neurons located on the antennae and the proboscis in the moth *Heliothis virescens*. Journal of Comparative Neurology 496:121-134

II. Jørgensen K., Almaas T.J., Marion-Poll, F., Mustaparta H. (2007). Electrophysiological characterisation of responses from gustatory receptor neurons of *sensilla chaetica* in the moth *Heliothis virescens*. Chemical Senses (under revision)

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Introduction

The chemical senses, gustation and olfaction, are present in virtually all organisms, and are in evolutionary context considered to be the oldest of the senses. In addition to their importance in aiding animals to find nutritious food and avoid toxic items, these senses play a major role in reproductive behaviour, kin recognition, social organisation, predator-prey relationship, and nest finding. Whereas the olfactory system has evolved for perceiving airborne molecules, the gustatory system has evolved for sensing molecules in liquids, crucial in the final acceptance or rejection of food or oviposition sites in insects. Phagostimulants like sugars elicit feeding, and bitter substances warn against ingesting toxins and cause rejection. Both responses are innate. However, learning by experience of the two categories of stimuli can modify these innate behaviours.

Detection of tastants has evolved differently in various organisms, depending on diet breadth and habitat. In vertebrates, gustation is limited to a few modalities, and mammals seem to be unable to distinguish different chemicals within one taste modality. Humans perceive the five taste modalities: sweet, salty, sour, bitter, and umami (the taste of glutamate). In general, sweet, umami, and low concentrations of salts elicit feeding, whereas bitter, sour and high concentrations of salts deter feeding. In insects, and particularly lepidopteran larvae, separate gustatory receptor neurons (GRNs) responding to substances perceived as one taste modality in mammals have been shown, indicating detection of a wider range of taste qualities. No sequence similarity of the gustatory receptor genes in insects and mammals has been found, suggesting different origins of the genes. In addition, the gustatory systems in the two groups of animals show anatomical differences; e.g., the mammalian gustatory organs are comprised of secondary sensory cells located in the oral cavity, whereas insects have primary sensory neurons in gustatory sensilla located at several appendages of the body.

The gustatory system

The mammalian gustatory system

The anatomy of the gustatory system differs across phyla. Mammals have epithelial derived taste cells organised in taste buds (50-150 taste cells per bud) located in papillae on the tongue (Lindemann 1996). Apically, the gustatory receptor cells have microvilli extending into a taste pore cavity, exposing the receptor cells to chemicals in the mouth. Three morphologically different papillae types (fungiform, foliate, and circumvallate) are

topographically arranged on the tongue. Although the gustatory cells are not neurons with axons, they can fire action potentials that amplify the depolarisation leading to neurotransmitter release in response to stimulation with tastants (Roper 1983). The activity is transmitted to gustatory afferent fibres at the proximal part of the taste bud. Each gustatory afferent contacts several gustatory receptor cells within and between taste buds and follows one of three cranial nerves (Smith and Davis 2000). The chorda tympani (CT) branch of the VIIth cranial nerve (nervus facialis) innervates taste buds at the anterior part of the tongue, the glossopharyngeal branch of the IXth cranial nerve (nervus glossopharyngeus) innervates the posterior part of the tongue, and the superior laryngeal nerve (SLN) branch of the nervus vagus (cranial nerve X) innervates the epiglottis and larynx. The three nerves relay gustatory information in a loose topographical manner to the nucleus of the solitary tract (NST) of the medulla; the CT fibres terminate rostrally to the glossopharyngeal fibres, and the glossopharyngeal fibres terminate rostrally to the SLN fibres with some overlap between the projection areas (Figure 1A). Second order gustatory afferents from the NST synapse on neurons of the parabranchial nucleus (PbN) of the pons. Neurons of the PbN project to the ventral posterior medial nucleus (VPM) of the thalamus, from which neurons project to the primary gustatory cortex. In addition, neurons of the PbN project to limbic structures like the lateral hypothalamus and the amygdala. In primates, gustatory information converges with olfactory information in the orbitofrontal cortex providing the perception of flavour (Rolls and Baylis 1994). In addition to these main gustatory areas along the ascending pathway, other areas are involved, like the hippocampus (Kobayakawa et al. 1999). The neurons in the ascending and the modulatory descending pathways form a complex network involved in coding and learning of gustatory information (Jones et al. 2006).

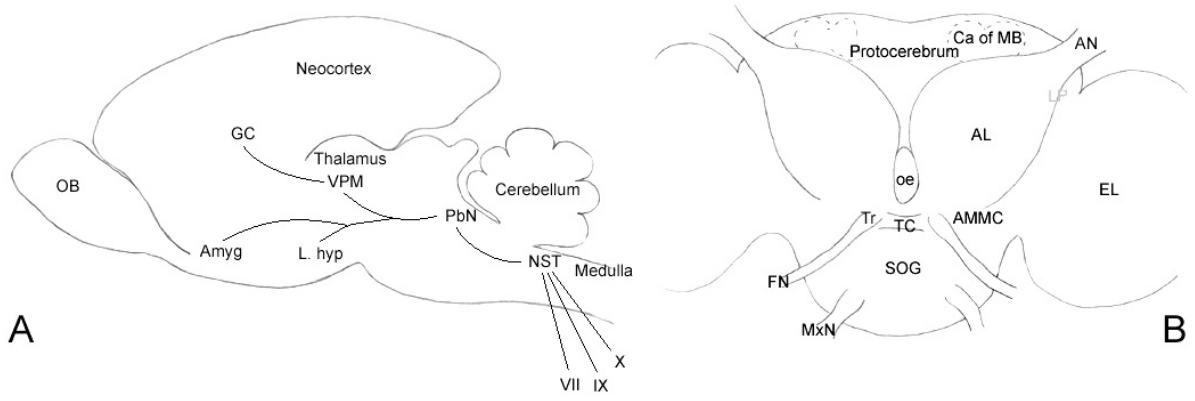

Figure 1*: Schematic overviews of the ascending gustatory pathways and some known chemosensory areas in the rat brain (A) and in the moth brain and SOG (B).* ***A:*** *The ascending gustatory pathway in the rat brain includes branches of cranial nerves VII, IX and*

X, mediating information from the taste buds on the tongue and in the oral cavity to the NST of the medulla. Second order gustatory afferents from the NST synapse on neurons of the PbN of the pons that relay information to the VPM of the thalamus, from which neurons project to the primary gustatory cortex. In a parallel pathway, neurons of the PbN project to the lateral hypothalamus and the amygdala. Amyg: amygdala, GC: gustatory cortex, L. hyp: lateral hypothalamus, NST: nucleus of the solitary tract, OB: olfactory bulb, PbN: parabrancheal nucleus, VII, IX and X: cranial nerves, VPM: ventral posterior medial nucleus. ***B:*** *Scheme of the H. virescens brain and SOG showing some gustatory and olfactory areas. The gustatory information from the proboscis projects via the MxN to the SOG/tritocerebrum. AL: antennal lobe, AMMC: antennal mechanosensory and motor centre, AN: antennal nerve, Ca: calyces, EL: eye lobe, FN: frontal ganglion nerve, LP: lateral protocerebrum, MB: mushroom bodies, MxN: maxillary nerve, oe: oesophagus, SOG: suboesophageal ganglion, TC: tritocerebral bridge, Tr: tritocerebrum.*

Both peripheral and central gustatory neurons in mammals have appeared relatively unselective to chemical types and typically respond to more than one (often three or four) of the taste modalities in addition to tactile and thermal stimuli (Smith and Shepherd 1999). However, when the response of one neuron to a specific substance was expressed as a proportion of the responses to the other substances, individual fibres of the CT nerve appeared as sucrose-best (S), NaCl-best (N) and HCl-best (H) fibres. The S fibres respond to substances like amino acids, sugars, and artificial sweeteners (Frank 2000). Information about sodium salts like NaCl is conveyed to the NST by two fibre types, the N and the H fibres. One third of the N fibres also respond to HCl. The H fibres are considered as generalists because they respond strongly to stimuli of several taste qualities (Smith and Davis 2000). In recent molecular biological studies, two families of gustatory receptor genes coding for the receptors, T1R and T2R, have been identified (Hoon et al. 1999; Adler et al. 2000). The dimer of T1R2 and T1R3 seems to detect all natural sugars and artificial sweeteners, whereas the dimer of T1R1 and T1R3 detects umami (Chandrashekar *et al.* 2006). For the coding of bitter, 25 T2R receptor types are involved in humans and 35 types in mice, and multiple bitter receptors are expressed in the same gustatory cells (Adler *et al.* 2000). Thus, unlike sugar-responsive cells detecting a large number of substances with one receptor complex, bitter cells detect a large diversity of bitter substances by many specialised receptor proteins expressed in single cells. In experiments where bitter receptors were expressed in sugar cells, stimulation with bitter substances resulted in phagostimulatory behaviour, demonstrating a hard-wired, labelled line arrangement from the gustatory receptor cells to the brain (Mueller *et al.* 2005). In future experiments, it will be interesting to see the results of studies combining molecular biology and physiology in mammals.

The transduction mechanisms for the five taste modalities, particularly sweet, umami and bitter, have recently been elucidated in molecular biological studies. Salt and sour are detected by ligand gated ion channels that open in the presence of cations that pass through and directly depolarise the cell membrane. The transduction pathways for bitter, sweet and umami, all seem to be G-protein coupled (Chandrashekar *et al.* 2006). Gustducin, a signalling molecule expressed selectively on the tongue, shows partially overlapping expression with the sweet, bitter and umami receptors (T1Rs and T2Rs) in gustatory cells. The same phospholipase C/ IP_3 second messenger pathway and cation channel (TRPM5) expressed selectively in gustatory cells seem to be involved in the transduction of all three modalities (Zhang *et al.* 2003). The neurotransmitter is suggested to be ATP (Finger *et al.* 2005). Discrimination of the three modalities in mammals is possible because different populations of gustatory receptor cells each express either sweet, bitter or umami receptors.

The insect gustatory system

Many insects have GRNs responding to the same tastants as mammalian receptors. However, depending on species and environment, the insect receptor neurons can in addition detect other substances. The contact chemosensilla (insect gustatory organs) are located on appendages, like antennae, tarsi, mouthparts, ovipositors and wings (De Boer and Hanson 1987; Ramaswamy 1988; Städler and Roessingh 1991; Stocker 1994; Baur et al. 1998; Chapman 2003). These sensilla consist of an outer hair shaped cuticular structure with a single pore at the tip, and an inner lumen containing 4-6 GRNs surrounded by three supporting cells (Schneider 1964; Steinbrecht 1984; Zacharuk 1985; Ozaki and Tominaga 1999). Different from the epithelial derived mammalian gustatory receptor cells, the GRNs in insects are primary sensory neurons with axons projecting to the CNS. The dendrites of the GRNs extend towards the tip pore of the sensillum hair where they are exposed to chemicals of the host plants or other materials when the sensillum is in contact with a substrate. In addition to the GRNs, many contact chemosensilla contain one mechanosensory receptor neuron with a dendrite attached to a cuticular structure at the base of the hair (Hallberg 1981; Chapman 1998; Ozaki and Tominaga 1999). In general, the axons of the GRNs project directly to the corresponding ganglia of the segment where they are located. GRNs on the mouthparts and some of the tarsal GRNs project to the suboesophageal ganglion (SOG, Figure 1B) and the tritocerebrum (Mitchell *et al.* 1999), whereas other tarsal and wing GRNs project to one thoracic ganglion (Stocker and Schorderet 1981; Rajashekhar and Singh 1994) and ovipositor GRNs in the terminal abdominal ganglion (Tousson and Hustert 2000). The

projections of antennal GRNs were not known in any species previous to the experiments included in this thesis. Because of the involvement of antennal GRNs in the proboscis extension reflex and in the association of olfactory and gustatory stimuli during learning it was of interest to study their projection patterns in the CNS.

Functional studies of GRNs in contact chemosensilla have been performed in many species since the pioneer work on the blowfly *Phormia regina* (Hodgson et al. 1955; Dethier 1955). Several extracellular recordings have shown that each GRN in a contact chemosensillum is specified for one taste modality and responds to many substances within the modality. However, the specificity of the neurons varies between species (Evans and Mellon jr. 1962; Blaney and Simmonds 1988; Simmonds et al. 1990; Chapman 1998; Schoonhoven and Van Loon 2002). In *P. regina* the sugar cell responds to sucrose, fructose, glucose, sugar alcohols, and some amino acids (Shiraishi and Kuwabara 1970; Dethier 1976), whereas in lepidopteran larvae, separate GRNs detect sugars, sugar alcohols and amino acids (Glendinning et al. 2000; Schoonhoven and Van Loon 2002). In addition, separate GRNs responding to a diverse range of deterrents, including substances that taste bitter to humans, have evolved in these insects (Dethier 1980; Schoonhoven *et al.* 1992). Bitter stimuli constitute the largest and structurally most diverse class of gustatory stimuli, being molecules with varying sizes and functional groups (Rouseff 1990). Previous to the experiments included in this thesis, physiological studies of antennal GNRs in adult insects had only been performed in honeybees, showing the presence of sucrose, but not bitter responses, in spite of a particular search for responses to bitter substances in one study (Haupt 2004; De Brito Sanchez et al. 2005). Thus, in our study of the moth *Heliothis virescens*, we wanted to find out whether the antennal GRNs responded to bitter substances as well as phagostimulants, or if the antennal sensilla were devoid of bitter GRNs like in the honeybee.

For insects as well as for mammals, recent molecular biological studies have enhanced the knowledge about taste recognition (Scott 2005). In fruitflies *Drosophila sp*, a divergent family of 68 putative 7-transmembrane candidate gustatory receptors has been identified (Clyne et al. 2000; Scott et al. 2001; Dunipace et al. 2001; Robertson et al. 2003). Whereas these genes share no sequence similarity to the mammalian T1R or T2R receptors, they show resemblance to olfactory receptors in insects, suggesting a common ancestor for the two chemosensory gene families. The Gr5a receptor in *Drosophila* is a candidate sugar receptor; genetic ablation results in behavioural taste deficits to trehalose, sucrose and glucose (Wang *et al.* 2004), and imaging studies show responses to sugars in the Gr5a projections (Marella *et al.* 2006). Another receptor gene, Gr66a, which is never co-expressed with the Gr5a gene, is

believed to code for a bitter receptor (Thorne *et al.* 2004; Wang *et al.* 2004). Genetic ablation of Gr66a results in behavioural taste deficits to bitter substances, but not to sugars, and Gr66a projections show responses to bitter substances in imaging studies (Marella *et al.* 2006). Various other gustatory receptors are co-expressed in subsets of Gr66a GRNs. Thus, activation of different subpopulations of GRNs, all containing Gr66a in addition to different combinations of other bitter receptors, provides a basis for discrimination between bitter tastants. In *H. virescens*, a candidate gustatory receptor gene (HR5) has been identified, which is expressed in cell bodies located at the base of the contact chemosensilla *sensilla chaetica* on the antennae (Krieger et al. 2002). However, the role of this receptor gene in gustation has not been functionally proven.

Learning and memory in insects

Classical conditioning in insects

In the animal kingdom, learning, remembering and forgetting are important mechanisms for adaptation to a changing environment. In feeding, learning and memory of the taste and smell of nutritious or noxious food is crucial for survival. Due to the relative simplicity of the insect nervous system with few, but fairly large, neurons insects have provided suitable model systems for studying the neural mechanisms and circuits behind complex behaviours like learning and memory (Menzel et al. 2006). Assays of physiology, biochemistry and behaviour have particularly been performed in the honeybee *Apis mellifera*, whereas molecular biological and behavioural methods have been combined in studies of *Drosophila*. The advantage of studying *Drosophila* is the known genome, which has enabled manipulation of genes, creating mutants with learning deficits, as well as determining what proteins are involved in learning and memory and their locations in the CNS. The most common learning paradigm in this species is to pair an odour stimulus with electric shock while another odour is presented without electric shock. In a subsequent choice test, the flies will show conditioned avoidance to the odour previously associated with electric shock.

The advantage of studying *A. mellifera* is its excellent ability to learn and remember. In nature, honeybees learn to associate colours, shapes and odours with nectar rewards followed by communication of this information to other members of the hive. In this species, learning of odorants has been studied in an easily controlled form of appetitive conditioning that involves the proboscis extension response (PER). When the GRNs on the antennae are stimulated with sucrose, the hungry honeybee extends its proboscis in order to feed (Bitterman et al. 1983; Menzel 1993; Hammer and Menzel 1995). If an odour (the

conditioned stimulus, CS) is given previous to the sucrose stimulation (the unconditioned stimulus, US), the bees learn to associate the odour with the sucrose reward, and the CS will subsequently trigger a conditioned response (CR), i.e. the honeybees extend the proboscis in response to the odour. The interval between the CS and the US should only be a few seconds for optimal learning. The predictive value of the CS is dependent on how reliable the US follows. Repeated stimulation with CS without US results in impaired subsequent learning, i.e., latent inhibition (Bitterman et al. 1983; Abramson and Bitterman 1986). This PER conditioning model of olfactory learning has provided a framework for studies of learning and memory in other insects (e.g., heliothine moths). Conditioning studies of these moths have shown that they are able to learn odours both in laboratory and in field experiments (Cunningham et al. 1999; Hartlieb et al. 1999; Skiri et al. 2005; Cunningham et al. 2006). Olfactory conditioning is particularly interesting to study in *H. virescens* because plant odorant receptor neurons are functionally characterised according to biologically relevant odorants; i.e., primary and secondary odorants have been identified (Mustaparta and Stranden 2005). Thus, when using the primary odorants in conditioning experiments, in principle only one type of olfactory receptor neuron is activated. Skiri et al (2005) found that conditioning with increased CS concentrations of the primary odorants increased the learning rates and the odorants activating different receptor neuron types caused different learning rates; i.e., they had different salience in *H. virescens*. However, the effect of increased US concentration in appetitive learning in *H. virescens* was not studied.

Neuronal pathways involved in olfactory conditioning

The olfactory pathways in insects have been described in many studies aimed at resolving the mechanisms involved in olfactory coding (Christensen and Hildebrand 1987; Boeckh and Tolbert 1993; Anton and Homberg 1999; Menzel and Giurfa 2001; Heisenberg 2003). The involvement of these pathways in olfactory conditioning has been the particular focus in studies of *A. mellifera* and *Drosophila* (Menzel and Giurfa 2001; Heisenberg 2003). In general, the odorants are detected by olfactory receptor neurons located in sensilla on the antennae (Schneider 1964; Steinbrecht 1999). Their primary axons form parts of the antennal nerve projecting to the glomeruli of the antennal lobe (AL, Figure 1B) (Homberg et al. 1989; Boeckh and Tolbert 1993; Berg et al. 1998; Vosshall et al. 2000). The glomeruli are condensations of synapses forming the neuronal networks between the sensory neurons and the AL interneurons; the local interneurons (mediating interglomerular inhibition) and the projection neurons (PNs) conveying information via three major antennocerebral tracts to the

protocerebrum (Homberg et al. 1988; Malun et al. 1993; Müller et al. 2002; Wong et al. 2002; Rø et al. 2007). Two major protocerebral areas receive olfactory information: the mushroom bodies (MBs), shown to be important in memory formation and storage, and the lateral protocerebrum (LP), a sensory-motor processing area providing a fast, but coarse odour analysis (Menzel and Giurfa 2001; Heisenberg 2003). Output MB neurons, like the PE1 neuron in *A. mellifera*, convey information to the LP. Modulation of olfactory responses during learning has been shown in this neuron (Rybak and Menzel 1998). An important neuron responsible for modulation during conditioning in *A. mellifera* is the ventral unpaired median neuron of the maxillary neuromere 1, VUM_{mx1}, with axonal arborisations that converge with the olfactory neurites in the ALs, the MBs and the LP, and dendrites converging with the gustatory pathways in the dorsal SOG and the tritocerebrum (Hammer 1993). Its functional role in connecting the pathways conveying information about the US and the CS has been demonstrated. Electrical stimulation of the octopaminergic VUM_{mx1} neuron in association with an odour puff was sufficient to replace sucrose reinforcement, suggesting that it comprises the neural substrate for sucrose reinforcement in *A. mellifera*. In addition, pairing of an odour stimulus with injection of octopamine in certain areas of the brain has shown that both the MBs and the ALs are involved in olfactory conditioning, presumably contributing to different aspects of learning (Hammer and Menzel 1998). Other octopaminergic VUM neurons recently discovered in the honeybee brain might also be involved (Schröter et al. 2007). In *H. virescens*, a bilateral symmetrical neuron similar to the VUM_{mx1} with the cell body in the midline of the SOG and extensive arborisations in the olfactory neuropil has been shown (Rø *et al.* 2007). However, the physiology of this neuron, as well as whether its dendrites converge with the axon terminals of the gustatory neurons is not known. In general, projections in the CNS have only been known for GRNs of the sensilla on the mouthparts and tarsi of insect species like *A. mellifera*, *P. regina*, *Drosophila*, the fleshfly *Neobellieria bullata,* and the desert locust *Schistocerca gregaria* (Edgecomb and Murdock 1992; Mitchell et al. 1999; Thorne et al. 2004; Wang et al. 2004). The studies of the present thesis and a parallel study by Kvello et al (2006) present projections of the GRNs on the antennae and the proboscis that are involved in appetitive learning of *H. virescens*.

Molecular mechanisms behind olfactory conditioning

A molecular model of olfactory conditioning in *Drosophila* has been made based on the molecular mechanisms of learning and memory in the sea slug *Aplysia californica* (Kandel and Abel 1995). The model suggests that PNs in *Drosophila,* mediating the CS, release

neurotransmitters that cause opening of Ca^{2+} channels in the postsynaptic Kenyon cells of the MBs, eliciting the following cascade: The weak influx of Ca^{2+} activates a Ca^{2+}/calmodulin dependent adenylyl cyclase (AC) converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) that in turn activates protein kinase A (PKA), which phosphorylates and closes K^+ channels, causing a weak depolarisation of the Kenyon cells. The US, mediated by a modulatory neuron, releases dopamine in aversive conditioning and octopamine in appetitive conditioning (Schwaerzel *et al.* 2003), activating a G-protein coupled receptor in the Kenyon cell. The G-protein activates AC, increasing the level of cAMP that causes K^+ channels to close. When the CS and the US are paired, both processes activate AC, which is the coincidence detector. This causes a prolonged closure of the K^+ channels and a broader, longer lasting action potential. The mobilisation of vesicles is augmented, inducing increased neurotransmitter release and resulting in a subsequent CR to the CS alone. In short, when the activation of the Kenyon cells representing an odour occurs simultaneously with the modulatory reinforcement signal, the output from the activated Kenyon cells to the MB output neurons is strengthened. Together, the studies of *A. mellifera* and *Drosophila* show the necessity of convergence of the CS and US pathways and coincidence detection at the cellular level in classical conditioning.

Memory processes

Memory develops over time after learning, and the consolidation and storing of memories are dependent on the environmental requirements of the insects. Changes of odour responses in the ALs and the MBs after olfactory conditioning have been demonstrated with optical as well as intracellular recordings in *A. mellifera* (Faber et al. 1999; Faber and Menzel 2001; Sandoz et al. 2003). With the exception of one study of *Drosophila* showing changes both in the ALs and MBs (Yu *et al.* 2004), most studies show that learning and memory is restricted to the MBs only (Gerber et al. 2004b). Insects have both short-term and long-term memory phases. When honeybees search for suitable foraging sites, an early short-term memory enables recognition of the nectar qualities of different plants within a flower patch, whereas a late short-term memory is used to remember nectar rewards between flower patches (Menzel 1999). These memory stages are transient, and sensitive to retrograde amnesia or additional experience (Erber 1976; Menzel 1990). If there has been only one learning trial, memory consolidates into a more stable and amnesia resistant middle term memory within approximately 1 h, declining over time (Menzel 1990). Only multiple spaced learning trials can lead to a stable long-term memory that does not decline over time. Two different types of

stable long-term memories have been described in the honeybee; a resistant early long-term memory, independent of protein synthesis and a protein synthesis (transcription) dependent late long-term memory (Wittstock *et al.* 1993; Wüstenberg *et al.* 1998). The same memory phases are found in olfactory conditioning of PER as in colour learning of free flying bees (Menzel 1999).

In addition to learning, insects have the ability to stop responding to cues that no longer provide a reinforcing (negative or positive) effect. Extinction is a decline in the CR when the learned CS is no longer reinforced (presented repeatedly without the US). The mechanism behind this phenomenon is unclear. One explanation is that it involves destruction of the original learning, as suggested in earlier studies of vertebrates (McClelland and Rumelhart 1985). However, some of the original learning seems to remain after extinction, as shown with spontaneous recovery, where an extinguished response recovers with the passage of time. This implies that extinction involves formation of a new memory that transiently inhibits the old one (Rescorla 2001; Bouton 2002). One study of honeybees suggests that extinction could instead reflect some destruction of the CS-US association, since spontaneous recovery is dependent on the number of conditioning trials and the interval between conditioning and extinction. Depending on training parameters, different memory substrates are affected by extinction, and spontaneous recovery can occur (Sandoz and Pham-Delegue 2004). An intracellular suppression of the old memory trace is shown in one study of *Drosophila*, in which extinction is an antagonistic process to the signalling cascade involved in associative memory formation (Schwaerzel *et al.* 2002).

Extinction as well as pre-exposure of the CS was utilised in two experiments included in the thesis to study the putative aversive effects of two bitter substances in *H. virescens*. Regular aversive conditioning is generally performed by exposing an animal to a chemical stimulus paired with food contaminated with nauseating effects that cause the animal to avoid the particular taste or smell. In our experiment, we wanted to find out whether bitter taste in itself was sufficient to create inhibitory learning and facilitate extinction in the moths. Due to the non-appetitive effect of quinine and sinigrin shown in papers I-II, a decrease in acquisition and memory was expected after exposure to these substances. Therefore it was necessary to optimise the conditioning parameters in order to produce higher learning performance in *H. virescens*. One experiment assaying retention and the stability of memory at different retention times was performed, as well as the one testing US concentration. These two studies allowed us to perform the bitter tastant experiments under optimised learning conditions.

Heliothis virescens

H. virescens (Insecta: Lepidoptera: Noctuidae) is a polyphagous species belonging to the subfamily Heliothinae that is comprised of numerous species living on all continents, several of them belonging to the most important pest species in agriculture. The larvae of *H. virescens* cause severe damage on monocultures of cotton, tomato, corn, soy beans, sunflower, and tobacco in North and South America (Fitt 1989; King and Coleman 1989). Their status as pest species is due to many factors, such as high polyphagy, mobility and fecundity, as well as the ability to have facultative diapauses and to resist insecticides. In addition, the feeding preference for reproductive and growing parts of the plants by the larvae causes severe damage to the plants that have fairly low damage thresholds. The female moths choose between many plant species for nectar feeding and oviposition, and they are attracted to the host plants by blends of odorants. However, in *H. virescens*, the final decision to feed or oviposit on a plant is made after antennating and ovipositor dragging on the leaf surface (Ramaswamy 1988). Taste substances on the plant surface and the composition of tastants in the nectar determine whether the plant is accepted. In addition, experience with the host plants seems to affect subsequent host plant choices in heliothine moths (Firempong and Zalucki 1991; Cunningham et al. 1998). *H. virescens* and other heliothine moths have been used as models in our laboratory to study olfactory coding and learning (Mustaparta and Stranden 2005). The pheromone system and detection of plant volatiles of *H. virescens* have been extensively studied, and narrowly tuned receptor neurons responding to primary and secondary odorants have been functionally classified (Almaas and Mustaparta 1990; Almaas and Mustaparta 1991; Berg and Mustaparta 1995; Berg et al. 1995; Røstelien et al. 2000a; Røstelien et al. 2000b; Stranden et al. 2002; Stranden et al. 2003a; Stranden et al. 2003b; Røstelien et al. 2005). Central olfactory pathways have been functionally as well as anatomically described (Christensen et al. 1991; Christensen et al. 1995; Mustaparta 1996; Rø et al. 2007). However, the gustatory pathways, as well as the physiology of the antennal contact chemosensilla in heliothine and other moths, remained unresolved when the studies of this thesis started. The ultimate goal of the studies included in this thesis was to contribute to resolving the neuronal networks involved in chemosensory coding and learning.

Aims of the thesis

The aims of the thesis were:

1. To morphologically characterise the contact chemosensilla *s. chaetica* on the antennae of *H. virescens* and trace the projections in the CNS of the receptor neurons of both *s. chaetica* and the proboscis *sensilla styloconica* (paper I).
2. To functionally characterise the receptor neurons of *s. chaetica* by testing mechanosensory stimulation and taste substances selected as physiologically relevant for *H. virescens* (papers I-III).
3. To study the behavioural significance of these tastants as phagostimulants or deterrents (papers I and II).
4. To study the putative aversive effect of two bitter substances in a conditioning context (paper III).
5. To enhance the learning and memory performances in *H. virescens* by studying parameters like US concentration and time (paper III).

Survey of the individual papers

Paper I

The aim of the study of paper I was to morphologically characterise the contact chemosensilla, *s. chaetica*, on the flagellum of the antennae of adult *H. virescens*, as well as to functionally classify the receptor neurons housed in the sensilla and determine the projection areas in the CNS of their primary axons. Scanning electron microscopy showed that each flagellar annulus, except the most distal, have 4-6 *s. chaetica* regularly distributed along transverse rows on the leading edge. The outer morphology of *s. chaetica* was characterized by a long, rigid hair with an annular surface pattern, a single pore at the tip, and a basal socket to which the hair shaft was attached.

Application of tetramethylrhodamine dextran to the receptor neurons of *s. chaetica* on the antenna resulted in stained axons that followed the antennal nerve to the entrance of the AL, bypassed the AL posterio-laterally, and projected ipsilaterally in two areas posterior to the tritocerebral commissure. The terminals showed a fan-shaped projection in the antennal mechanosensory and motor centre (AMMC) located posterior and ventral to the AL, and a finger-like projection reaching in a posterior-medial direction into the dorsal SOG. In the preparations where single sensilla were stained, 1-5 neurons could be identified in the CLSM images. Intensive staining obtained by applying dye to the cut flagellum showed substantial staining in the AL (due to staining of olfactory receptor neurons), the AMMC, and the SOG. The projection areas of the receptor neurons of several *s. chaetica* were similar to the projection areas of the single sensillum receptor neurons, but covered a larger area. In some individuals, the receptor neurons of *s. chaetica* on the left flagellum and of the contact chemosensilla *s. styloconica* on the proboscis (left galea) were stained. These preparations showed projections in two separate, but closely located areas in the tritocerebrum/dorsal SOG, posterior to the tritocerebral commissure. Axons of the receptor neurons of the gustatory sensilla on the proboscis entered the tritocerebrum/ SOG via the maxillary nerve and terminated alongside, but anterior-medially, to the terminals of the antennal gustatory neurons. No overlap of the projection areas of the receptor neurons on the two appendages was found, neither when staining single nor several sensilla.

The results obtained from electrophysiological recordings demonstrated the presence of one mechanosensory and 3-4 gustatory neurons in *s. chaetica*. Recordings with tungsten microelectrodes at the sensillum base showed no spontaneous activity of the neurons. In tip

recordings, nearly all sensilla had GRNs responding to sucrose, while the responses to KCl, leucin, sinigrin and water differed between sensilla, independent of their location on the flagellum. In experiments using the PER, stimulation of GRNs in *s. chaetica* with sucrose elicited a vigorous response by proboscis extension, whereas a non-appetitive response appeared when stimulating with sinigrin.

Paper II

The study of paper II was aimed at characterising the GRNs of *s. chaetica* according to their responses to selected substances considered as relevant tastants for the adult *H. virescens*. Tip recordings included systematic surveys of concentration series of KCl, sucrose, inositol, NaCl, sinigrin, quinine, and ethanol. In general, excitatory phasic-tonic firing was recorded, except for the response to quinine (0.001 M) that showed excitatory bursts of spikes at irregular intervals. Based on spike analysis and response profiles of individual sensilla, it appeared that sucrose and quinine activated separate GRNs, whereas the responses to KCl, NaCl, sinigrin, inositol, and ethanol were more difficult to ascribe to particular GRNs. The phagostimulant sucrose and the two bitter substances quinine and sinigrin elicited responses in the largest proportion of the GRNs of *s. chaetica*. Variations of sensitivity were observed between the GRNs in different sensilla, both in respect to threshold concentrations and response strength. Highest sensitivities were found for the sucrose-responsive GRNs and the quinine-responsive GRNs. For the other substances, the GRNs showed relatively low sensitivities. The GRN composition within individual sensilla varied to a great extent, and no systematic distribution of particular sensillum types was found.

The variation of the response profiles appeared as follows. Responses to the two phagostimulants, sucrose and inositol, were obtained both in the same and in separate sensilla. Similarly, responses to the two bitter substances as well as the two inorganic salts appeared within single and separate sensilla. Using spike analyses some general features appeared. The spikes of the GRNs responding to sucrose were broader than those of the other GRNs. The GRNs responding to KCl, NaCl, inositol, and sinigrin had smaller spike amplitudes than the GRNs responding to sucrose, water, quinine, and ethanol. The GRN responding to quinine showed a gradual increase in spike amplitude during a burst, and the response to sinigrin differed from the quinine response both in spike amplitude and temporal firing pattern. Another GRN, probably a water responsive GRN, appeared with large spikes and tonic firing during stimulation with the lowest concentration of all substances. The spikes of this GRN usually disappeared at higher concentrations, when the other GRNs were activated.

Comparisons of responses to sucrose and mixtures of sucrose and quinine or sinigrin were performed in order to study possible interactions between phagostimulatory and deterrent GRNs. The average firing of the sucrose GRNs decreased with increasing concentrations of quinine or sinigrin in the mixture with sucrose. In addition, the bursting response to quinine disappeared when stimulating with the mixture, implying a mutual inhibition of the GRN responses to sucrose and quinine. These series of stimulations with single compounds and mixtures of sucrose and quinine or sinigrin imply that both sinigrin and quinine act excitatory on separate GRNs and cause inhibition of the sucrose responsive GRN. The behavioural significance of the phagostimulant sucrose and the putative deterrent quinine was assayed by applying quinine and sucrose to the antennae of a group of 30 moths. When quinine was applied to the antennae, only one moth extended its proboscis. In the subsequent stimulation with sucrose on the antennae of the same group of insects, 21 moths extended their proboscises, implying that quinine is non-appetitive to *H. virescens*.

Paper III

The aim of the study of paper III was to assay the putative aversive effects of the two bitter substances, quinine and sinigrin, on the adult moth *H. virescens* in an olfactory conditioning context. These two substances were in electrophysiological recordings shown to elicit excitatory responses, probably in two separate GRN types of *s. chaetica* (papers II and III). In addition, both quinine and sinigrin was found to induce a non-appetitive effect in the moths (papers I and II).

Two main protocols were used to investigate the aversive effects of the two tastants. In the first protocol (pre-exposure), two groups of moths were pre-exposed to the odour linalool (CS) paired with one of the tastants, whereas the control group was pre-exposed to the linalool CS and a mechanosensory stimulus. A fourth group of moths was not pre-exposed. In the subsequent acquisition phase, the moths treated with quinine in the pre-exposure phase showed a higher resistance to acquisition than the control group. Treatment with sinigrin showed a similar effect as treatment with quinine, but the difference from the control group was not significant. The control group showed significantly lower acquisition than the group of untreated moths, indicating a latent inhibition phenomenon in the control group.

In the second protocol (facilitated extinction), moths were first subjected to an acquisition phase with CS and sucrose, before being subjected to an extinction phase, where the same CS was associated with one of the tastants, quinine or sinigrin, or with no tastant (control). Extinction both in the quinine and the sinigrin groups was faster than in the control

group, implying that both quinine and sinigrin facilitated extinction of the conditioned response compared to the unrewarded presentations of linalool. The results of the experiments with pre-exposure and facilitated extinction indicated a latent inhibition effect, as well as an aversive effect of quinine and to a lower extent, of sinigrin. The results also suggested that the two tastants may act as negative reinforcers in *H. virescens*.

Due to the non-appetitive and putatively aversive effects of the bitter substances (papers I and II), a decrease in CRs in these kinds of experiments was expected. Therefore the learning rates of the moths had to be improved before the pre-exposure and facilitated extinction experiments described above were carried out. Conditioning with both 2 M and 3 M sucrose as US induced good acquisition, without any differences between the two concentrations. Retention was not affected by the US molarity, but by the time elapsed after training. Comparing the first extinction trial performed 15 min, 2 h, 8 h, 24 h, and 48 h after training in different groups of moths showed that memory decreased with time, being strongest at 15 min and declining gradually to a lower level at 48 h. The strength of the odour-sucrose association at different times after conditioning was studied by comparing its resistance to extinction during 8 extinction trials. In all cases the responses decreased with increasing number of extinction trials. The moths tested after 8 h showed the fastest and highest overall extinction, whereas the 48 h group showed a slower and lower overall extinction than the other groups, suggesting a consolidation of memory within 48 h.

Discussion

GRN specificity and sensitivity

The results of papers I-III contribute to the knowledge about gustation in insects, particularly about the anatomical and functional properties of antennal gustatory sensilla. Although the presence of contact chemosensilla on the antenna has been known from earlier studies of many species, their functional significance has not been assayed, except in two studies of the sucrose responsive GRNs on the honeybee antennae (Haupt 2004; De Brito Sanchez et al. 2005). Most studies, including those of *H. virescens*, have focused on the GRNs of contact chemosensilla located on mouthparts, ovipositors, or tarsi (Blaney and Simmonds 1988; Blaney and Simmonds 1990; Chapman 2003). Comparison of the morphology of these sensilla on different appendages, including *s. chaetica* on the antennae of *H. virescens* (paper I), shows the typical properties of a hair/peg formed outer cuticular structure, with a uniporous hair tip, and a basal socket, as described in many early and recent studies (Schneider 1964; van der Peers et al. 1980; Steinbrecht 1984; Zacharuk 1985; Ozaki and Tominaga 1999; Kvello et al. 2006; Jørgensen et al. 2006). The number of 4-6 GRNs housed in the contact chemosensilla on the different appendages is relatively constant in the various species (Hallberg 1981; Koh et al. 1995; Ozaki and Tominaga 1999), including *s. chaetica* of *H. virescens* with 4 GRNs as shown in TEM studies (Færavaag 1999) and the present thesis (paper I). Commonly, each GRN of a contact chemosensillum has been considered as specified for one tastant. However, depending on diet and habitat, the specificity differs in various species (Evans and Mellon jr. 1962; Blaney and Simmonds 1988; Simmonds et al. 1990; Chapman 1998; Schoonhoven and Van Loon 2002).

In all animals, detection of phagostimulants and deterrents is particularly important due to their nutritional and toxic values, respectively. This is reflected in separate GRNs for sugars and bitter tastants, as shown in electrophysiological studies of insect contact chemosensilla (Dethier 1976; Blaney and Simmonds 1990; Glendinning J.I. and Hills 1997; Bernays and Chapman 2000; Hiroi et al. 2002; Chapman 2003; Meunier et al. 2003; Thorne et al. 2004; Haupt 2004). The significance of these taste modalities in heliothine moths is shown in paper II, indicating separate GRNs specified for sucrose and the two bitter substances quinine and sinigrin, respectively. In contrast to the strong responses to these tastants, the weak and unspecific responses, as well as the high thresholds to KCl, NaCl, inositol, ethanol, and leucin suggest a minor role of these substances in feeding. Possibly, other tastants present

in plants play more significant roles for *H. virescens*. Absence of biologically relevant tastants in the selection of test substances might have caused the impression of variability and unspecific responses, disabling a classification of distinct sensillum types. This particularly applies to GRNs of varying sensitivities, a feature known in insect GRNs as well as in sensory fibres of mammals (Schoonhoven 1976; Smith and Shepherd 1999) that contributes to the intensity coding, extending the detectable concentration range of the tastants.

Feeding animals, including herbivorous insects, encounter complex mixtures of nutrients and other items. Many plant species contain toxins, and detection of the relative amount of toxic to nutritious substances is important for acceptance or rejection of food in herbivorous insects. In general, activity in the phagostimulatory GRNs stimulates feeding whereas activity in the deterrent GRNs inhibits feeding. Thus, acceptance or rejection of a potential food source depends on the ratio of activity in the two populations of GRNs. In particular, sucrose and other sweet tastants stimulate cells that ultimately connect to neurons controlling the ingestion of nutritive substances, whereas quinine and other aversive stimuli affect neural systems controlling rejection reflexes that prevent the ingestion of toxic substances. The present thesis shows that activation of sucrose GRNs elicits proboscis extension and feeding in *H. virescens* whereas activity of the quinine and sinigrin GRNs results in inhibition of proboscis extension, and thus feeding. The inhibitory effect caused by sinigrin has previously been shown in *H. virescens* larvae where the amount of food consumed was clearly negatively correlated with the firing rate of the sinigrin-responsive GRNs (Bernays et al. 2000; Bernays and Chapman 2000). In general, attractive and aversive behaviours in response to chemicals are found in all organisms, from simple forms like the bacteria *Escherichia coli* with only five chemosensory receptor proteins (Fain 2003) to mammals. In mammals, CT nerve fibres are relatively more responsive to phagostimulants (sugars and salts) than the fibres of the glossopharyngeal nerve that are more responsive to aversive stimuli (acids and bitter). Afferent input from the CT nerve is important for ingestive behaviour, while input from the glossopharyngeal nerve is important for rejection (Smith and Shepherd 1999). In the rat, sucrose and quinine produce opposite patterns of ingestive and aversive behaviour, respectively (Grill and Norgren 1978). Thus like in *H. virescens*, input about tastants seems to be directly related to a specific pattern of behavioural reaction in mammals, as also demonstrated in the molecular biological study showing a hard-wired arrangement from the sweet and bitter receptor cells to the neurons controlling behaviour (Mueller et al. 2005). The link between identified GRNs and their behavioural significance is also demonstrated in *Drosophila* where activation of the sugar receptor Gr5a and the bitter

receptor Gr66a seem to be sufficient for mediating acceptance and avoidance, respectively (Marella et al. 2006).

The relative firing of phagostimulatory and deterrent GRNs should be sufficient to signal what is eatable or not in an organism. Interestingly, an additional mechanism of mutual inhibition has evolved, attenuating the response of the GRN mediating opposite information. This might facilitate the aversive or stimulatory behaviours linked to the deterrent and phagostimulatory tastants, respectively. Inhibitory interactions of information between the two categories of tastants are observed in the responses of insect GRNs as well as mammalian fibres of the CT and PbN (Schoonhoven et al. 1992; Smith et al. 1994; Formaker and Frank 1996; Chapman 2003). In particular, the suppression of sucrose responses by quinine seems to be a widespread phenomenon (Dethier and Bowdan 1989; Chapman et al. 1991; Dethier and Bowdan 1992; Formaker et al. 1997; De Brito Sanchez et al. 2005). In insects, as shown in the present study of *H. virescens* (paper II), quinine inhibits firing of the sucrose GRNs when stimulating with a mixture of the two substances. This is in accordance with a previous behavioural study showing increased inhibition of PER during stimulation of tarsal contact chemosensilla with mixtures of sucrose and increasing concentrations of quinine (Ramaswamy et al. 1992). Similar behavioural studies, assaying PER responses to stimulation of antennal contact chemosensilla with mixtures of sucrose and bitter substances are topics of future experiments. Inhibition of bitter responses by sucrose is also seen in insects and mammals. In *H. virescens* (paper II), no spikes from the quinine responding GRNs appeared when stimulating with the mixture of sucrose and quinine. In hamsters, sucrose stimulation suppresses quinine responses in the PbN (Smith et al. 1994). Thus it seems that mutual inhibition is an important feature in processing information about phagostimulants and deterrents in insects and in vertebrates.

When assaying the vast results of electrophysiological recordings from the afferent fibres and brain areas involved in gustation combined with the molecular and behavioural studies performed in many species, it seems that the coding of the gustatory information, for example in labelled line versus across fibre patterns is a matter of interpretation. As discussed above, activity in certain gustatory neurons might be sufficient for coding of the palatability of food, suggesting some kind of a labelled line system. The hard-wired system demonstrated in the molecular biological studies supports the principle of a labelled line system in mammals (Mueller et al. 2005). However, this does not exclude the possibility of a supplemental across fibre pattern arrangement. Another molecular biological study, knocking out the T1R3 receptor involved in both sweet and umami taste demonstrated that the mice

could detect and discriminate the two tastants without this particular receptor (Delay et al. 2006). This implies that additional receptors are involved in sweet and umami taste. It is likely that both mechanisms of labelled line and across fibre pattering take part in gustatory coding. In mammals, the ascending fibres with best responses to specific taste modalities may be involved in a labelled line system, whereas the generalist H fibres may contribute in an across fibre manner. However, these two models might not be sufficient to explain the complexity of the gustatory system, as discussed in a recent review (Jones et al. 2006). Modulation plays an important role, as shown in experiments in which bitter substances that originally caused aversion mediated appetitive behaviour after repeated stimulation when no nauseating effects were experienced with the substance. Responses to gustatory stimuli can be modulated at several levels in the gustatory system in mammals, from the peripheral receptor cells to the highest order of gustatory neurons in the brain.

Organisation of the central pathways

Proximity of gustatory and mechanosensory neurons

The presence of gustatory and mechanosensory neurons in the same sensory organs, mediating information about texture and gustatory quality is a feature appearing throughout the animal kingdom (Rolls 2004). In humans, the texture of food is mediated by mechanosensory fibres in the oral cavity, giving an additional dimension to the food quality during mastication. In herbivorous insects, the mechanosensory information from the external contact chemosensilla informs about the physical contact with and the structure of the plant surface and particular food source, whereas the mechanosensory information from the internal sensilla possibly concerns viscosity of the food or simply elicitation of the swallowing reflex. In mammals, the oral mucosa and lingual epithelium are innervated by general somatosensory fibres mediating information via the cranial nerves V, IX and X about touch as well as pain and temperature (Smith and Davis 2000). In addition, single peripheral gustatory fibres can respond to tactile and thermal stimuli as well as gustatory stimuli (Smith and Shepherd 1999), all mediating important information about food. The proximity of the sensory neurons detecting gustatory and mechanosensory stimuli is preserved as the information is conveyed to higher order neurons in the CNS in mammals (Smith and Davis 2000). More than half of the taste responsive neurons in the NST receive input from both gustatory and tactile receptors. Further anatomical proximity is shown by the lingual branch of cranial nerve V terminating in the gustatory portion of the NST, overlapping rostrocaudally with the gustatory inputs of the CT fibres. A rough topographic representation of the oral cavity with

overlapping gustatory and somatosensory receptor fields is also shown in the VPM. In the gustatory cortex, the oral somatosensory input seems to be located immediately dorsal to the area receiving gustatory input.

The presence of mechanosensory neurons in contact chemosensilla, like in *s. chaetica* of *H. virescens* (paper I), is common in all insect species (Hallberg 1981; Koh et al. 1995; Ozaki and Tominaga 1999). A general problem in the anatomical studies has been to unambiguously separate the mechanosensory and gustatory fibres of the contact chemosensilla, as discussed in paper I. However, the one large diameter axon of the receptor neurons of contact chemosensilla in both *P. regina* and *H. virescens* (paper I) is assumed to belong to the mechanosensory neuron (Edgecomb and Murdock 1992; Jørgensen et al. 2006). As shown in paper I, the large diameter axon of the presumed mechanosensory neuron projects to the AMMC, whereas the small diameter axons of the GRNs project to the SOG, two neighbouring areas in the moth CNS (Figure 1). In addition, some preparations indicated an overlap of the projection patterns, such that projections of the thick and thin fibres were found in both areas. Projections to the AMMC and the SOG of mechanosensory neurons and to the SOG of gustatory neurons are also found in other insect species (Suzuki 1975; Strausfeld 1976; Hildebrand et al. 1980; Koontz and Schneider 1987; Homberg et al. 1989; Rehder 1989; Mitchell and Itagaki 1992; Stocker 1994; Kloppenburg 1995; Mitchell et al. 1999; Thorne et al. 2004). This implies that the proximity of mechanosensory and gustatory information is preserved from the peripheral gustatory organs to higher CNS areas in insects like in mammals, showing the significance of the associated mechanosensory and gustatory information during feeding.

Central gustatory projections

Whereas some knowledge exists regarding the integration of gustatory information and functional organisation of the gustatory pathways in mammals, such data are only scarcely reported in insects. Intracellular recordings of local SOG interneurons in the fleshfly *Sarcophaga bullata* have shown separate neurons responding to labellar stimulation with sucrose and salt (KCl), with the sucrose neuron also responding weakly to water (Mitchell and Itagaki 1992). Bitter substances were not tested. In locusts, recordings from thoracic local interneurons have been performed, showing responses to all of the test substances: sucrose, NaCl, lysine glutamate and nicotine hydrogen tartrate. No interneurons or motor neurons responded specifically to one of the chemicals. In *H. virescens*, intracellular recordings combined with staining have started, with the aim to study how the information about the

different gustatory stimuli is handled by second order neurons, as well as how the gustatory pathways are functionally organised (Kvello et al, 2007). Prerequisite for these studies are results obtained in the present thesis (papers I-III), defining the primary gustatory areas in the SOG/tritocerebrum receiving information from the GRNs on the antennae and proboscis as well as the closely located area, AMMC, receiving information from the associated mechanosensory receptor neurons of *s. chaetica* on the antennae. In addition, the GRN tuning, particularly to sucrose and the two bitter substances quinine and sinigrin, is important for resolving how the information is transmitted from the periphery to the CNS.

The organotopic organisation in the CNS of GRNs located on different appendages in insects is interpreted with respect to their different functional roles. Similar to the projections in separate CNS areas of the GRNs on the antennae and proboscis in *H. virescens*, GRNs on the mouthparts and legs of *Drosophila* show different projection patterns in the SOG (Wang et al. 2004). In *H. virescens*, the distinct localisation of the two areas (paper I) may reflect the functional differences of the two types of gustatory sensilla. Whereas GRNs of the *s. chaetica* are involved in the antennating behaviour during the search for food and the extension of the proboscis, the *s. styloconica* GRNs are involved in ingestion of food and proboscis recoiling. The role of *s. chaetica* was demonstrated in the behavioural experiments in papers I-III in which stimulation of the sensilla with sucrose led to proboscis extension, and stimulation with sinigrin or quinine inhibited the uncoiling of the proboscis. In addition, stimulation of the *s. styloconica* with sucrose led to increased ingestion. Thus, the two sensillum types seem to be involved in different behaviours, making the topographic separation of their projections reasonable. In contrast, no topographical organisation of the flagellar GRN projections appeared neither in respect to the GRN location on the flagellum nor to taste modality. The four GRNs of the same *s. chaeticum* ran tightly together and projected within the same area. In mammals, a crude topographic organisation of gustatory stimuli is observed in the NST (Smith and Shepherd 1999). The neurons from the fungiform papillae on the anterior part of the tongue mediating mainly sucrose and NaCl information, project caudally to the fibres mediating sour and bitter information from the foliate and circumvallate papillae on the posterior two-thirds of the tongue. This slight anatomical segregation continues throughout the gustatory pathway to the cortex. Recently, molecular labelling of gustatory afferents has shown a certain segregation of neurons providing information about sweet and bitter in the NST, the PbN, the VPM, and the gustatory cortex (Sugita and Shiba 2005). In *Drosophila*, molecular biological studies have shown separation of GRN projections mediating sweet and bitter (Marella et al. 2006). The molecular biological experiments in *H. virescens* have so far

identified one putative gustatory receptor protein (HR5), expressed at the base of *s. chaetica* on the antennae (Krieger et al. 2002). However, the expression pattern of the appurtenant receptor neuron axons in the CNS is not known. Future experiments combining molecular biological tools and physiological recordings may resolve the projection patterns of the GRNs mediating different taste modalities in *H. virescens*.

Olfactory conditioning with sucrose and bitter tastants

The importance of sugars is not only reflected in their phagostimulatory characters, but also in their role as positive reinforcers in appetitive conditioning of odorants in various species. Oppositely, the bitter substance quinine has been shown to act as a deterrent and a negative reinforcer in some species. Conditioned inhibition of the proboscis extension to sucrose in adult *Drosophila* has been observed when PER elicited by sucrose was punished by applying quinine to the fore tarsi (DeJianne et al. 1985). In addition, quinine is aversively associated with olfactory or other gustatory stimuli in this species (Mery and Kawecki 2002). Differential conditioning of bumblebees has shown that quinine acting as a negative reinforcer enables the insects to discriminate between visual stimuli faster than if the CS was paired with an absence of reward (Chittka et al. 2003; Dyer and Chittka 2004). In contrast to these studies, quinine was found to have an aversive, but not a reinforcing effect in associative learning in *Drosophila* larvae (Gerber et al. 2004a; Hendel et al. 2005).

The two bitter substances quinine and sinigrin studied in this thesis elicited different temporal firing patterns in the GRNs (papers II and III), suggesting a differentiation by the GRNs of the two substances. The behavioural experiments (papers I and II), showed that both substances were non-appetitive, but no aversive effects could be measured in the PER experiments since the moths either extended their proboscides or not, disabling the study of a negative response. To find out whether quinine and sinigrin were aversive, we used PER conditioning experiments, already established in the lab (Skiri et al. 2005). We studied the putative aversive effects of quinine and sinigrin using pre-exposure and facilitated extinction experiments. In the pre-exposure experiments in paper III, only quinine was shown to be aversive, although a clear tendency appeared for sinigrin as well. In this experiment it was also interesting that pre-exposure to linalool (paired with the dry toothpick) caused significantly reduced learning performance in the acquisition phase compared to untreated moths. It is possible that this group showed a typical latent inhibition phenomenon, described in a number of animals, like honeybees (Abramson and Bitterman 1986; Chandra et al. 2001). During repeated presentations of CS in the absence of a punishment or a reward, the CS might

be associated with the absence of reinforcement, leading to a resistance towards re-learning the CS as a predictor for a reward (or punishment) in the subsequent acquisition phase. The lower learning in the acquisition phase can also be due to learned inattention in which the CS becomes less and less surprising throughout the pre-exposure phase, and therefore looses meaning (Lubow, 1997). When the CS (paper III) was paired with quinine or sinigrin in the pre-exposure phase, the acquisition deficit was further increased, although the effect was not significant for sinigrin. Possibly, the moths built aversive associations between linalool (CS) and quinine as an aversive negative reinforcer. Thus, at the end of the pre-exposure phase, linalool stimulation predicted the presence of a negative stimulus, which had a stronger obstructing effect on acquisition than just an absence of a reward or punishment. Although our experiments showed that quinine had an aversive effect in moths, a definite proof for a negative reinforcing effect of quinine is still lacking, since we did not control for possible non-associative effects of quinine. Future experiments including a pre-exposure phase where moths receive unpaired presentations of CS and the bitter substances will constitute a control for the formation of aversive CS-bitter associations.

The results of the facilitated extinction experiments in paper III showed that both quinine and sinigrin enhanced extinction. Again the results might be explained by the formation of aversive associations. The moths would then learn two associations after one another; during acquisition, they would form CS-sucrose associations acting positively on PER, and during the second phase, they would form CS-quinine or CS-sinigrin associations, causing a resistance to elicit PER. The responses would reflect a balance between the two types of associations, the aversive association overbalancing the appetitive association. The second type of explanation could be that increased extinction with the bitter substances is a form of operant learning, because PER was punished by providing the bitter substance to the antennae and the proboscis.

If quinine and sinigrin are negative reinforcers in *H. virescens*, we expect that the reinforcement signals triggered by quinine and sinigrin will converge with the olfactory pathway to form associations, possibly involving a modulatory neuron with an opposite effect to the VUMmx1 in honeybees. In honeybees (Vergoz et al. 2007) and in *Drosophila* (Schwaerzel et al. 2003), dopamine has been found to be the neurotransmitter involved in aversive olfactory learning with electric shock as punishment. Moreover, in *Drosophila* larvae, activation of dopaminergic neurons in association with an odour stimulus was sufficient to create an aversive olfactory memory (Schroll et al. 2006). Independent of whether quinine or sinigrin are negative reinforcers or not, the pre-exposure and facilitated

extinction experiments show that both quinine and sinigrin are aversive and thus behaviourally relevant deterrents for *H. virescens*.

Memory phases in H. virescens

In order to influence performance and adaptability of an organism, the learned information must be remembered and the consolidation and storing of memories are dependent on environmental requirements. Learning of plant odorants in moths serves self consumption and oviposition purposes, so a strong memory shortly after learning declining over time as shown in *H. virescens* (paper III) may be well adapted to the life of the moth. The 15 min and 2 h memories can be equivalent to the late short-term memory phase described in *A. mellifera*, developing over time in the minute range, and used to remember rewards (nectar quality and quantity) between flower patches (Menzel 1999). In honeybees, this memory stage is transient and sensitive to retrograde amnesia or additional experience, which fits well with the little resistance to extinction in the 15 min and 2 h groups of *H. virescens* (paper III) (Erber 1976; Menzel 1990). The moths tested after 48 h showed low retention but a strong resistance to extinction, suggesting that the CS-US association was strong and stable in the moths that remembered the odour. Two different types of stable long-term memory have been described in other insects. A resistant form of memory, independent of protein synthesis, is found in the early long-term memory in honeybees as well as the anaesthesia-resistant memory in *Drosophila*. The second type is the protein synthesis (transcription) dependent late long-term memory that is formed after 3-4 days in *A. mellifera*. Whether the 48 h memory in *H. virescens* is a protein synthesis dependent long term memory will have to be investigated in future studies.

Conclusions and future prospects

This thesis constitutes the first steps in assaying the gustatory system in *H. virescens*. The results have shown that several tastants are detected by antennal GRNs, and that sucrose and bitter substances are especially important, eliciting strong responses in the GRNs on all parts of the flagellum. In addition, sucrose is shown to be highly appetitive to the moth *H. virescens*, whereas the bitter substances act as deterrents and are both non-appetitive and aversive, possibly acting as negative reinforcers in the appetitive conditioning context. One mechanosensory neuron is also present in each *s. chaetica*. The gustatory and mechanosensory neurons project to the SOG/tritocerebrum and the AMMC, respectively. GRNs of the proboscis and the antennae project to closely located but separated areas in the CNS, suggesting that the two appendages provide different information to the moth brain. Conditioning experiments show that *H. virescens* has a long-term memory that is resistant to extinction.

Future studies involving molecular biology, intracellular recordings and calcium imaging of second order neurons in the SOG and protocerebrum may show how the gustatory information is transmitted and processed in the CNS of *H. virescens*. In particular, it will be interesting to find out whether information about phagostimulants is mediated by different second order neurons than information about deterrents, or whether both types of information are integrated in some neurons of the gustatory pathways. Intracellular recordings may further enable the revelation of modulatory connections from the gustatory to the olfactory neuropils involved in appetitive and aversive learning. Calcium imaging experiments may demonstrate changes of activity in the AL and MBs during associative appetitive and aversive learning in *H. virescens*.

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Individual papers

Paper I

Paper 1 is not included due to copyright.

Paper II

Electrophysiological characterisation of responses from gustatory receptor neurons of *sensilla chaetica* in the moth *Heliothis virescens*

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Abstract

Discrimination of edible and noxious food is crucial for survival in all organisms. We have studied the physiology of the gustatory receptor neurons (GRNs) in contact chemosensilla (insect gustatory organs) located on the antennae of the moth *Heliothis virescens*, emphasising putative phagostimulants and deterrents. Sucrose and the two bitter substances quinine and sinigrin elicited responses in a larger proportion of GRNs than inositol, KCl, NaCl, and ethanol, and the firing thresholds were lowest for sucrose and quinine. Variations in GRN composition in individual sensilla occurred without any specific patterns indicating specific sensillum types. Separate neurons showed excitatory responses to sucrose and the two bitter substances quinine and sinigrin, implying that the moth might be able to discriminate bitter substances in addition to separating phagostimulants and deterrents. Besides being detected by separate receptors on the moth antennae, the bitter tastants were shown to have an inhibitory effect on phagostimulatory GRNs. Sucrose was highly appetitive in behavioural studies of proboscis extension, whereas quinine had a non-appetitive effect in the moths.

Key words: Antennal taste, sucrose, quinine, sinigrin, proboscis extension, insect taste

Introduction

Gustation is an omnipresent sense in virtually all organisms, and is used in finding and securing the quality of food, as well as avoiding toxic items. In selecting food and oviposition sites, female insects use gustatory receptor neurones (GRNs) located in contact chemosensilla on various parts of the body (De Boer and Hanson 1987; Ramaswamy 1988; Städler and Roessingh 1991; Bernays and Chapman 1994; Baur et al. 1998; Chapman 2003). In the moth *Heliothis virescens* (Lepidoptera: Noctuidae), contact chemosensilla are located on the antennae (*sensilla chaetica*), the proboscis (*sensilla styloconica*) and the tarsi (Blaney and Simmonds 1990; Kvello et al. 2006; Jørgensen et al. 2006). A contact chemosensillum typically contains 2-4 GRNs with dendrites extending towards the tip of the sensillum hair, and one mechanosensory neurone attached to the hair base (Hallberg 1981; Koh et al. 1995; Ozaki and Tominaga 1999; Kvello et al. 2006), and the antennal *s. chaetica* has 4 GRNs and one mechanosensory neuron (Jørgensen et al. 2006). When the moth antennates, gustatory stimuli are detected by the GRNs of *s. chaetica* that are especially abundant at the antennal tip. Information from the antennal GRNs is conveyed by their primary axons to the

suboesophageal ganglion (SOG) (Jørgensen et al. 2006), where it is transmitted to interneurones and motorneurones involved in the proboscis extension reflex (PER). Phagostimulants like sucrose, applied to the antennae, release PER when the moth is hungry and motivated to feed, whereas deterrents inhibit the release of PER. During feeding, GRNs on the proboscis are stimulated and convey information to the tritocerebrum/SOG (Kvello et al. 2006), controlling ingestion. Despite the importance of antennal GRNs in feeding, few studies of these neurons have been performed.

Detection of tastants has evolved differently in various organisms, depending on diet breadth and habitat. Mammals detect only a few taste modalities, and seem unable to distinguish different chemicals within each taste category, whereas insects seem to differentiate between a wider variety of tastants, including substances within categories. Sugars, an important energy source, are detected by particular gustatory cells, present in many species. In mammals, the two coupled receptor proteins, T1R2 and T1R3, seem to detect all natural sugars and artificial sweeteners tested (Chandrashekar et al. 2006). The specificity of the insect GRNs involved in sweet taste vary between species (Evans and Mellon jr. 1962; Blaney and Simmonds 1988; Simmonds et al. 1990; Chapman 1998; Schoonhoven and Van Loon 2002). In the blowfly *Phormia regina*, one sugar-responsive GRN responds to all of the feeding stimulants, sucrose, fructose, glucose, sugar alcohols, and some amino acids (Shiraishi and Kuwabara 1970; Dethier 1976), whereas separate GRNs detect sugars, sugar alcohols, and amino acids in lepidopteran larvae (Glendinning J.I. et al. 2000; Bernays and Chapman 2000; Schoonhoven and Van Loon 2002). The fleshfly *Boettcherisca peregrina* evidently has ionotrophic sugar detection (Murakami and Kijima 2000), whereas the transduction mechanism in *P. regina* and the fruitfly *Drosophila* involves a G-protein coupled cascade reaction with cGMP as second messenger (Amakawa and Ozaki 1989; Amakawa et al. 1990; Thorne et al. 2004). A putative sugar receptor, Gr5a, has been identified in *Drosophila* (Dahanukar et al. 2001). In *H. virescens*, a candidate gustatory receptor gene is expressed in cell bodies located at the base of *s. chaetica*, but the specificity of the receptor is not known (Krieger et al. 2002).

In addition to detecting phagostimulants, most animals, including herbivorous insects, possess GRNs responding to a diverse range of deterrents (Dethier 1980; Schoonhoven et al. 1992). Bitter stimuli constitute the largest and most structurally diverse class of gustatory stimuli (Rouseff 1990). In mammals, a family of gustatory receptors, T2R, is involved in bitter taste detection (Adler et al. 2000). Approximately 30 T2R receptor types are present in humans and mice, and multiple bitter receptors are expressed in the same gustatory cells

(Mueller et al. 2005). In *Drosophila*, the receptor gene, Gr66a, is believed to code for a bitter receptor (Thorne et al. 2004; Wang et al. 2004). In addition, various other putative bitter receptors are co-expressed in subsets of Gr66a neurons, implying that several types of GRNs mediate bitter taste. Thus, unlike sugar-responsive cells detecting many substances with one receptor type, bitter cells detect a large number of bitter substances with several receptor types expressed in the same cell, both in mammals and insects. This might provide a mechanism enabling flies to discriminate between bitter tastants possibly eliciting different behaviours. In insects, little is known about the transduction mechanisms behind detection of bitter tastants. In *P. regina*, a lipophilic ligand-binding protein has been found to carry lipophilic members of toxic taste substances to the deterrent GRNs (Ozaki et al. 2003), and a ligand-gated GABA/ glycine chloride channel has been found in the western corn rootworm, *Diabrotica virgifera* (Mullin et al. 1994). One previous study assaying antennal detection of bitter by GRNs in the honeybee *Apis mellifera* revealed an absence of such GRNs on the antennae (De Brito Sanchez et al. 2005).

The moth *H. virescens*, a serious pest on monocultures like cotton, tomato, corn, soy beans, grain, and tobacco (Fitt 1989; King and Coleman 1989) is a polyphagous species also preferring other host plants. The females choose between many plant species for nectar feeding and oviposition. The moths are attracted to the host plants by blends of odorants, but the final decision to feed or oviposit is made after antennating and ovipositor dragging on the leaf surface (Ramaswamy 1988). Taste substances on the plant surface and the composition of taste substances in the nectar determine whether the plant is accepted. In the present paper assaying the physiology of the GRNs on the antennae of female *H. virescens*, we have focused on the following substances of putative importance in host plant selection. The sugar sucrose is present in high levels in Lepidoptera-pollinated plant nectar (Baker and Baker 1983), the sugar alcohol *myo*-inositol is detected by specialised GRNs in *H. virescens* larvae (Bernays and Chapman 2000), the alcohol ethanol is observed to be attractive to *H. virescens* larvae, KCl and NaCl are two important inorganic salts, and quinine and sinigrin are known as bitter substances. The alkaloid, quinine, is found to act through blocking certain K^+ channels in vertebrates, or to permeate cell membranes directly and activate G-proteins, bypassing the receptor in *in vitro* preparations (Spielman et al. 1992; Naim et al. 1994). The glucosinolate sinigrin is found to be non-appetitive for *H. virescens* and other lepidopterans (Blaney and Simmonds 1988; Shields and Mitchell 1995b; Jørgensen et al. 2006). The aim of the present study was to functionally characterise the antennal GRNs in respect to specificity and sensitivity to these substances of putative importance to female *H. virescens*. In addition, we

wanted to study GRN composition in the different *s. chaetica* to find out if it was similar or different across sensilla.

Material and methods

Insects and preparation

H. virescens used in the experiments were received as pupae (Novartis Crop Protection AG, Rosental, Switzerland). The male and female pupae were sorted and hatched with access to 5% sucrose solution in separate climate chambers (Refritherm 200, Struers-Kebolab, Albertslund, Denmark; 22°C, reversed photoperiod). On the day of the experiment, the adult female moths (1-2 days old) were immobilized with tape and wax between the head with the thorax in Plexiglas holders, exposing the head and the antennae. The antennae were attached to a wax foundation with tungsten hooks so that the leading edge was facing upwards making the *s. chaetica* accessible.

Test substances

The gustatory stimuli used in the experiments were (applied in the following order) KCl, sucrose, the sugar alcohol *myo*-inositol, NaCl (all from Sigma-Aldrich), the glucosinolate sinigrin monohydrate, the alkaloid quinine hydrochloride (both from VWR), and ethanol (Arcus) prepared in dilutions of the electrolyte 0.01 M KCl. The concentration range was from 0.0001 M to 0.1 M for KCl, sucrose, inositol, NaCl, and sinigrin (up to 1 M for NaCl). Quinine was applied at two concentrations only (0.00001 M and 0.001 M) due to putative damage of the cells by this substance. Ethanol was applied at 5% (1 M), 10% (2.2 M), and 20% (4.3 M). Studies of GRN interaction were performed with mixtures of 0.01 M sucrose and quinine (0.00001 M and 0.001 M) or 0.01 M sucrose and sinigrin (0.01 M and 0.1 M). The experiments started with the lowest concentrations and ended with the highest to avoid adaptation in the cells. The solutions were prepared every two weeks and stored at 4°C. For the behavioural experiments 1.0 M sucrose and 0.16 M quinine dissolved in distilled water was used.

Electrophysiology

Electrophysiological recordings from GRNs of *s. chaetica* were carried out using tip recording (Hodgson et al. 1955). The recording electrode (thin walled borosilicate glass

capillaries, Harvard apparatus) was pulled in a two step electrode puller (PP-830, Narishige group, Japan) to a tip diameter of approximately 10-20 µm. To avoid crystallisation and concentration changes at the tip, the electrode was filled with the test substance just a few seconds before the start of the recording. The recording electrode containing the test solution was placed over single sensilla hairs for five seconds with an inter stimulus interval of approximately 10 minutes to avoid adaptation. The recording glass electrode was connected to a TastePROBE amplifier (10x, Syntech, Hilversum, Netherlands) (Marion-Poll and Van der Peers 1996) and the signals were filtered (low pass: 50 Hz and high pass 3000 Hz) using CyberAmp 320 (Axon Instruments). The grounded reference electrode was a 1 mm diameter AgCl coated silver wire placed in the moth abdomen or in the contralateral eye. Analyses of the spikes were performed using the software AutoSpike-32 (Syntech). The annuli were numbered 1-81 from the most proximal to the most distal annulus of the flagellum, and recordings were made from the four sensilla on each annulus without preferences. All sensilla between annulus 81 and 55 were described as distally located, between 54 and 27 medially located, and between 26 and 1 proximally located. Only the three highest concentrations (0.001 M, 0.01 M, and 0.1 M) were included in the dose-response curves to avoid interference of the water cell that was firing at 0.0001 M.

Statistics

For each substance, the proportion of GRNs responding to the substance distally, medially and proximally on the flagellum was compared using Fisher's exact tests. Differences in response strength distally, medially and proximally on the flagellum were compared using Kruskal-Wallis tests, and when applicable, 2-by-2 comparisons were performed using Mann-Whitney tests.

Behaviour

In order to compare behavioural effects of the appetitive stimulus sucrose and the putative aversive stimulus quinine, PER experiments were performed. Moths were starved for 24 h before they were tested for PER by applying 0.16 M quinine or 1.0 M sucrose to the antennae. In the first part of the experiment, quinine was applied to the moth antennae, and the number of proboscis extensions was counted. After 10 minutes, sucrose was applied to the antennae of the same moths, and the number of proboscis extensions was compared to that elicited by quinine.

Results

Proportion of *s. chaetica* with GRNs responding to the test substances

The results are based on electrophysiological recordings from 132 *s. chaetica* of 11 moths, systematically tested for concentration series of the following seven substances: KCl, sucrose, inositol, NaCl, sinigrin, quinine, and ethanol. The GRNs that fired in a dose-response manner to a particular substance were considered to be responsive to the substance. In general, excitatory phasic-tonic firing was recorded as responses to all stimuli (Figures 1A,E, 2A,E, 3A,E, 4A,E), except for 0.001 M quinine that elicited an excitatory bursting firing at irregular intervals (Figure 1A). The latency of the cell responding to quinine varied and sometimes extended four seconds. The most active substances were quinine, sucrose, and sinigrin eliciting GRN responses in a larger proportion of *s. chaetica*; quinine in 74% (98 of 132), sucrose in 65% (85 of 130), sinigrin in 46% (60 of 131), KCl in 39% (48 of 124), NaCl in 35% (24 of 84), ethanol in 31% (29 of 95), and inositol in 25% (32 of 128). Complete recordings at all concentrations were missing in some sensilla, causing the difference in numbers of tested sensilla. The distribution of these GRNs differed along the flagellum (Figures 1C,G, 2C,G, 3C,G, 4C), the proportion of sensilla with GRNs responding to sucrose increased significantly from the base to the tip of the flagellum (All parts: Fisher's exact test, $p < 0.001$; 2-by-2 comparisons by Fisher's exact tests: Distal vs medial, $p = 0.5$; Distal vs proximal, $p < 0.001$; Medial vs proximal, $p = 0.005$), whereas the opposite was observed for KCl (All: Fisher's exact test, $p < 0.019$; 2-by-2 comparisons by Fisher's exact tests: Distal vs medial, $p = 0.83$; Distal vs proximal, $p < 0.011$; Medial vs proximal, $p = 0.032$). The number of GRNs responding to the other substances was approximately equal along the flagellum (Fisher's exact tests, $p > 0.544$ in all cases). → **Figures 1-4**

Sensitivity of the GRNs

The sensitivity varied between the GRNs in different sensilla, both in respect to threshold concentrations and response strength. Quinine, the only substance tested at 0.00001 M, elicited responses in 51% of the quinine responsive GRNs at this concentration. The average firing frequency was 2.9 imp/ s, increasing to 18.2 imp/ s at 0.001 M (Table 1, Figure 1B). Due to the bursting firing, the response to quinine is given as imp/ s during the bursting period. At higher concentrations than 0.001 M, quinine caused noise, and the spikes disappeared in all GRNs within the sensilla. Even hours after stimulation with higher concentrations of quinine, the recordings showed only irregular noise to the other test

substances. Therefore, tests with quinine were only performed twice in each sensillum at concentrations causing no damage. Like the quinine responsive GRNs, the GRNs responding to sucrose showed a high sensitivity; all activated by 0.001 M sucrose with an average firing frequency of 18.8 imp/ s (Table 1, Figure 2B). At the highest concentration of sucrose (0.1 M), the average firing frequency was 66.8 imp/ s, the strongest average response measured (Table 1, Figures 1B,F, 2B,F, 3B,F, 4B). The individual sensitivities of these GRNs showed variations from 3 to 133 imp/ s as responses to 0.1 M sucrose. The other test substances had higher threshold concentrations than 0.001 M (Table 1, Figures 1B,F, 2B,F, 3B,F, 4B), and the dose-response curves showed an overall lower response to these substances compared to sucrose. → **Table 1**

Differences in the GRN response strength to the individual substances were evident along the flagellum. Sucrose, quinine and ethanol elicited significantly stronger responses distally and proximally than medially on the flagellum (Figures 1D, 2D, 4D) (Sucrose (all parts): Kruskal-Wallis, $p = 0.019$; 2-by-2 comparisons, Mann-Whitney: Distal vs medial, $p = 0.03$; Proximal vs medial, $p = 0.02$; Distal vs proximal, $p = 0.119$; Ethanol (all parts): Kruskal-Wallis, $p = 0.010$; 2-by-2 comparisons, Mann-Whitney: Distal vs medial, $p = 0.026$; Proximal vs medial, $p = 0.007$; Distal vs proximal, $p = 0.310$; Quinine (all parts): Kruskal-Wallis, $p = 0.001$; 2-by-2 comparisons, Mann-Whitney: Distal vs medial, $p < 0.0001$; Proximal vs medial, $p = 0.019$; Distal vs proximal, $p = 0.395$). Inositol elicited significantly stronger firing proximally than distally (All parts: Kruskal-Wallis, $p = 0.07$; 2-by-2 comparisons, Mann-Whitney: Distal vs medial, $p = 0.388$; Proximal vs medial, $p = 0.277$; Distal vs proximal, $p = 0.019$) (Figure 2H), whereas KCl, NaCl and sinigrin elicited approximately equal firing at all parts of the flagellum (Kruskal-Wallis, $p > 0.207$ in all cases).

Comparison of responses between individual *s. chaetica*

In 76 sensilla, complete recordings were obtained at all concentrations of each substance. Comparison between the individual response profiles of the sensilla showed variations (Table 2). Separate sensilla showed responses to the two inorganic salts in two populations of 18 and 16 sensilla, respectively, whereas 12 other sensilla showed responses to both salts. The two bitter substances also elicited responses in different sensilla, 29 only to quinine and 8 only to sinigrin, while 29 others showed responses to both. In addition, individual variations were observed between responses to sinigrin and the two salts; GRNs in 13 sensilla responding only to KCl, in 21 only to sinigrin, and in 16 to both. Fifteen sensilla showed responses only

to NaCl, 24 only to sinigrin, and 12 to both. In addition, responses to the two phagostimulants sucrose and inositol showed individual variations between sensilla; in 37 sensilla responses appeared only to sucrose, in 10 only to inositol, and in 9 to both. Comparison between inositol and ethanol showed 6 sensilla with responses to both, 14 only to inositol and 15 only to ethanol. → **Table 2**

Analysis of single GRN responses

Spike analysis were performed in order to separate spikes originating from different GRNs. Overall, a definite identification of the neuron types across recordings was difficult due to the change of recording electrodes with varying conductance. In spite of this, some general features appeared. The GRNs responding to KCl, NaCl, inositol, and sinigrin had smaller spike amplitudes (less than 1 mV) than the GRNs responding to sucrose, water, quinine, and ethanol (Figures 1A,E, 2A,E, 3A,E, 4A,E, 5). The relative large spikes of the GRNs responding to sucrose were broader than those of the other cells (Figures 2A, 5). The GRN responding to quinine showed a gradual increase in spike amplitude during a burst, and the response to sinigrin differed from the quinine response both in spike amplitude and temporal firing pattern (Figures 1A,E, 5B). Concerning the two salts, two GRNs with different spike amplitudes seemed to be involved in the responses to both KCl and NaCl (Figure 5). Figure 5A shows activity of the small amplitude GRN, and figure 5B of the larger amplitude GRN. Firing of both as well as of only one of them appeared in the recordings. The small amplitude GRN fired vigorously to 0.1 M and 1 M NaCl, whereas the large amplitude GRN often displayed a low frequency firing at all NaCl concentrations. Stimulation with KCl showed a similar response pattern. Variations considered not to be real responses were occasionally seen in different recordings, as exemplified in figure 3E (third trace) where a large amplitude GRN appeared, that did not fire to the other concentrations of NaCl. Peculiarly, the response to ethanol consistently showed larger spike amplitudes (2 mV) at the highest concentration than at the two lowest (Figure 4A), possibly due to the fat-soluble properties of ethanol. Another GRN, probably a water responsive GRN, appeared with large spikes and tonic firing during stimulation with the lowest concentration (0.0001 M) of all substances (Figure 4E, upper trace), and occasionally to 0.001 M (Figure 2E, upper trace). The spikes of this GRN usually disappeared at higher concentrations, when the other GRNs were activated. In a few cases where no excitatory response to the test substance was observed, this water GRN showed decreased firing with increasing concentration of the substance, exemplified in figure 4E.

The different compositions of GRNs in individual sensilla are exemplified in figure 5. Both recordings show responses to sucrose, NaCl, KCl, and inositol. Responses to sinigrin and ethanol are evident in the recordings shown in figure 5A, whereas response to quinine is seen only in the recordings shown in figure 5B. Based on the analysis of spike amplitudes and wave forms, it seems that the response to KCl, NaCl and sinigrin originate from the same GRN; in figure 5A from the small amplitude GRN and in figure 5B from the larger amplitude GRN. The characteristic broad spikes are elicited by the sucrose GRN, whereas the spikes elicited by inositol originate from a third GRN. In addition, the largest spikes in the two recordings originate from an ethanol GRN (Figure 5A) and a quinine GRN (Figure 5B), respectively. → **Figure 5**

Responses to mixtures of sucrose and bitter substances

Comparisons of the responses to sucrose and the mixtures of sucrose and the two bitter substances, quinine and sinigrin, were performed in order to study possible interactions between phagostimulatory and deterrent GRNs. Stimulation with mixtures of sucrose and quinine were performed in 92 sensilla with separate GRNs responding to sucrose and quinine (Figure 6A-C). The average responses to the initial and final stimulation with 0.01 M sucrose were approximately equal, 54 and 53 imp/ s, respectively. The average firing decreased to 39 and 14 imp/ s, respectively, when 0.00001 M and 0.001 M quinine was mixed with the 0.01 M sucrose solution. In addition, the bursting response to quinine was not seen when quinine was mixed with sucrose, implying a mutual inhibition of the quinine- and sucrose-responsive GRNs. The GRN responding to quinine had a long and inconsistent latency when stimulated with quinine alone, whereas the latency of inhibition of the sucrose responsive GRN was immediate, impairing the sucrose response from the start of the stimulation period.

In 44 other sensilla, sinigrin elicited the same pattern of inhibition when stimulating with the mixtures of 0.01 M sucrose and two different concentrations (0.01 and 0.1 M) of sinigrin (Figure 6D-F). Because of sensitivity differences, higher concentrations of sinigrin than quinine were used in the mixtures. The initial and final stimulation with sucrose elicited an average firing of 41 imp/ s, whereas the mixtures with increasing concentrations of sinigrin elicited decreased firing (27 and 9 imp/ s). These series of stimulations with single compounds and mixtures of sucrose and the two bitter substances imply that both sinigrin and quinine act excitatory on separate neurons and cause inhibition of the sucrose responsive GRN. → **Figure 6**

Behaviour

Behavioural effects of the phagostimulant sucrose and the putative deterrent quinine was assayed by applying 0.16 M quinine and 1.0 M sucrose to the antennae of 30 starved moths. When quinine was applied to the antennae, only one moth extended its proboscis. In the subsequent stimulation with sucrose on the antennae of the same group of insects, 21 moths extended their proboscises, showing that sucrose is highly appetitive whereas quinine is non-appetitive.

Discussion

The results in the present study have shown that the moth *H. virescens* has GRNs responding to all seven selected tastants, with strongest responses to sucrose and quinine. In addition, sucrose- and quinine-responsive GRNs were present in a majority of the *s. chaetica*. However, the GRN composition of individual sensilla varied to a great extent, showing no distinct sensillum types or distribution of specific types to particular locations. This absence of sensillum types might appear because of the limited number of test substances as well as varying sensitivities of the GRNs. Other biologically relevant tastants might have elicited stronger responses, particularly in the weakly activated GRNs. The varying sensitivities of the GRNs might have enhanced the impression of variability of the responses, disabling a classification of sensillum types.

We based our choice of test substances on their statuses as general phagostimulants or deterrents as well as expected relevance to *H. virescens*. Sucrose, an important energy source and the most prominent component in the nectar of Lepidoptera- pollinated plants (Baker and Baker 1983), is a well known phagostimulant and relevant for *H. virescens* during nectar feeding, as evidenced by the strong responses in numerous GRNs in our study. When the moth searches for food or oviposition sites, it antennates, tapping the surface rapidly with the antennal tip. Approaching a flower, the whole flagellum of *H. virescens* is in contact with the interior of the flower, whereas the tip is touching the nectar source. This behaviour in combination with the vital importance of sugar might be reflected in the relatively large number of specific sucrose responding GRNs at the antennal tip, also found in other insects (Dethier 1976; Blaney and Simmonds 1990; Hiroi et al. 2002; Thorne et al. 2004; Haupt 2004). The second expected phagostimulant, the sugar alcohol inositol, is ubiquitous in plants, a key structural component of phospholipids, involved in osmoregulation and phosphate

storage in animals, as well as being a second messenger probably in all insects (Loewus 1990). Its phagostimulatory effect is well known in many insect species, including the tobacco hawkmoth *Manduca sexta* (Dethier 1976; Bernays and Chapman 1994; Chapman 2003). We found no evidence for a general phagostimulatory GRN type responding both to sucrose and inositol, as reported in the fleshfly *Sarchophaga bullata* (Shimada 1987). The different spike shapes of the responses, as well as responses to only one of them in some sensilla, indicated that separate GRNs were activated by the two substances. Overall, the weak firing of few GRNs during stimulation with inositol, imply that no specific inositol GRN was present in these moths. In contrast, Lepidopteran larvae, including *H. virescens*, have GRNs vigorously responding to inositol (Dethier and Kuch 1971; Shields and Mitchell 1995a; Schoonhoven et al. 1998; Bernays and Chapman 2000), implying that ingestion of inositol is more important for larvae than adults, although both need inositol due to its overall importance in the cells. One might speculate whether the nectar of the host plants is devoid of inositol, while it is present in leaves, explaining the absence of specialised inositol GRNs in adults. In addition, inositol might be more vital to growing and developing larvae than to adults, or adults synthesise inositol easier than larvae, diminishing the need to acquire it through ingestion.

As putative deterrents we selected quinine and sinigrin. The prototypical bitter substance, the alkaloid quinine, is used in studies of many organisms, and the glucosinolate, sinigrin, is a non-appetitive tastant for *H. virescens* and other lepidopterans (Blaney and Simmonds 1988; Shields and Mitchell 1995b; Jørgensen et al. 2006). In a recent study of adult *H. virescens*, we have shown an aversive effect of both quinine and sinigrin in a conditioning context (Jørgensen *et al.*, submitted). As shown in the present study, the bitter substances were detected by specific GRNs, corresponding to results obtained in studies of other insect species (Glendinning J.I. and Hills 1997; Bernays and Chapman 2000; Chapman 2003; Meunier et al. 2003; Thorne et al. 2004). The presence of bitter GRNs on insect antennae has not previously been found, in spite of particular search for them on the antennae of honeybees (De Brito Sanchez et al. 2005). Separation of the responses by the two quinine and sinigrin GRN types in *H. virescens* was based on the different response patterns, bursting and phasic-tonic, respectively, as well as responses to only one of the substances in some sensilla (Fig 1, Tab 2). The bursting activity with long latency elicited by quinine in the GRNs is previously described in several insect species (Dethier 1980; Chapman et al. 1991; Schoonhoven et al. 1992). In humans, a long latency of the perception of bitter taste is known, which is proposed to be caused by a slow and long lasting binding to the receptor (Rouseff 1990). An alternative interpretation of the responses to quinine and sinigrin in the present

study might be that they originate from the same GRN, where the different temporal response patterns result from the involvement of two receptor types and possibly different excitatory transduction pathways, as suggested in *M. sexta* (Glendinning J.I. and Hills 1997). Co-expression of different bitter receptor proteins in the same GRN is shown in molecular studies of *Drosophila* (Thorne et al. 2004; Wang et al. 2004). Having several receptor types for different bitter substances in subsets of bitter responsive GRNs increase the ability of the insect to discriminate the components in mixtures of bitter substances in plants, and allow differentiation between toxic and harmless constituents, possibly eliciting different behaviours of acceptance or rejection. The behavioural experiments showed a non-appetitive effect of both quinine and sinigrin in this study as well as in Jørgensen et al. (2006), in contrast to the highly appetitive effect of sucrose in both studies. Possibly, there is a hard-wired labelled line arrangement from the gustatory receptors to the brain driving the two different behaviours, as shown in mammals, by expressing bitter receptors in sugar gustatory cells, resulting in phagostimulatory behaviour towards bitter substances (Mueller et al. 2005).

In nature, feeding animals, especially herbivores, encounter complex mixtures of nutrient and other substances. The responses of the GRNs are thus greatly affected by interactions between chemicals (Schoonhoven et al. 1992; Smith et al. 1994; Chapman 2003). In particular, the suppression of phagostimulant GRN activity by bitter substances, e.g. quinine, is a widespread phenomenon in several species (Dethier and Bowdan 1989; Chapman et al. 1991; Dethier and Bowdan 1992; Formaker et al. 1997; De Brito Sanchez et al. 2005). In the present study, quinine and sinigrin caused excitatory responses of particular GRNs as well as inhibition of the sucrose- and water- responsive GRNs (Fig 6), similar to the results obtained from GRNs on the prothoracic legs of *Drosophila* (Meunier et al. 2003). Feeding is positively correlated to activity in phagostimulatory GRNs, and negatively correlated to activity in deterrent GRNs, suggesting that quinine and sinigrin inhibit feeding both by exciting the deterrent GRNs and inhibiting the sucrose GRNs in *H. virescens* moths. In *H. virescens* larvae, a clear negative correlation has been found between the firing rate of the sinigrin-responsive GRNs and the amount of food consumed (Bernays et al. 2000; Bernays and Chapman 2000). In addition, behavioural studies of adult *H. virescens*, assaying PER during tarsal stimulation, showed an increasing inhibition of PER when stimulating with mixtures of sucrose and increasing concentrations of quinine (Ramaswamy et al. 1992). How the two kinds of information (phagostimulatory and aversive) is transmitted to second order neurones in the insect CNS, e.g. VUM-like neurones or motorneurons is an interesting question in future studies. In our study, there also seemed to be an inhibition of the bitter-

responsive GRNs by the sucrose GRN, since no spikes from these GRNs were observed when stimulating with the mixture. This kind of mutual inhibition is also observed in the parabranchial nucleus in hamsters (Smith et al. 1994). In addition, we demonstrated interactions between the water-responsive GRNs and the GRNs responding to the test substances. Suppression of water-responsive GRNs by other substances is previously shown in the fly *Phormia terranovae* (Rees 1970). In our study, neither quinine nor sinigrin caused any damage to the GRNs, shown by the similar firing to the initial and final sucrose stimulation.

The two inorganic salts, KCl and NaCl, are in general important in regulating the osmotic equilibrium in all organisms. K^+ is the major cation in plants, and present in high concentration in lepidopteran haemolymph (Dethier 1977). The responses to the inorganic salts in our study seem to originate from two GRNs eliciting small and large spike amplitudes, respectively. The GRN that often fired vigorously with small amplitude spikes to high salt concentrations might be the same GRN responding to sinigrin. Several GRN types involved in the response to inorganic salts, as well as deterrent receptors detecting high concentrations of salts are previously reported in other insects (Dethier and Hanson 1968; Bernays and Chapman 2001; Chapman 2003; Hiroi et al. 2004; Marella et al. 2006). In our study, the GRNs fired weakly to low salt concentrations and often vigorously to higher concentrations, which might influence feeding behaviour, eliciting feeding or avoidance, respectively, as shown in an early study of the blowfly (Dethier 1968). This seems to be reflected in the nutritional needs; low levels of salts being satisfactory, whereas high concentrations threaten the osmotic equilibrium. There is no evidence that insects ever suffer from salt deficiency in nature, possibly reflected by the weak overall salt responses. The stronger average firing to KCl than to NaCl might reflect the moths' common exposure to KCl in plants. We expected the two salts to be detected by the same GRN type, but some sensilla had GRNs responding to only one of the salts, indicating involvement of separate GRNs in salt detection. In contrast, two types of channels in the same GRN accepts different cations in *Drosophila* (Siddiqi et al, 1989), suggesting a possible discrimination of salts by the same GRNs.

Ethanol was included because according to our observations, it seems to be attractive to the *H. virescens* larvae. The highest concentrations of ethanol and quinine elicited peculiar response properties, ethanol causing tonic firing of larger spikes than at lower concentrations, and quinine spikes of increasing amplitude during the bursts. Possibly, high concentrations of these substances act on the GRN membranes. One *in vitro* study of the amphiphilic quinine have shown that it permeate cell membranes directly, bypassing the receptors, and activate G-

proteins (Naim et al. 1994). Ethanol is fat-soluble, and might also act directly on the GRN membranes, causing large amplitude spikes. However, ethanol did not elicit responses in the sucrose or inositol-responding GRNs, in contrast to recordings from monkey chorda tympani nerves showing that ethanol stimulate sweet-best fibres, and at high concentration some salt-best fibres (Hellekant et al. 1997).

Recordings from *s. chaetica* in the present study showed responses to more than four substances in each sensillum (Fig 5). Since *s. chaetica* have only four GRNs, it implies that at least one GRN responded to more than one substance, like the mammalian afferent gustatory fibres (Smith and Davis 2000). However, specific GRNs responding to sucrose and quinine were found, which by activation elicited appetitive and non-appetitive behavioural responses, respectively. Like in mammals, this might be a hard-wired arrangement where phagostimulants and deterrents elicit different innate behaviours.

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Figure legends

Fig 1: Response properties of GRNs in *s. chaetica* of *H. virescens* responding to the two bitter substances sinigrin and quinine. A: Responses and spike analyses of three different sensilla to quinine, illustrating the variation of the bursting response to 0.001 M quinine. The spike amplitude increased during bursts. (The response properties of the sensillum in the two upper traces to other substances are shown in figure 5B). B: Dose-response curve of average firing to quinine. C: Distribution of quinine-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $p > 0.05$). D: Response strength to 0.001 M quinine of the GRNs located along the flagellum. Different letters indicate significant differences (Mann-Whitney tests, $p < 0.05$). E: Example of responses and spike analyses of one GRN to sinigrin. There was no response to 0.001 M. (The response properties of this sensillum to other substances are shown in figure 5A). F: Dose-response curve of average firing to sinigrin. G: Distribution of sinigrin-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $p > 0.05$). H: Response strength to 0.1 M sinigrin of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann-Whitney tests, $p > 0.05$).

Fig 2: Response properties of GRNs in *s. chaetica* of *H. virescens* to sucrose and the sugar alcohol inositol. A: Example of responses and spike analyses of one GRN to sucrose. Sucrose elicited spikes with relatively high amplitude and broad spike shape. B: Dose-response curve of the average firing to sucrose. C: Distribution of sucrose-responding GRNs along the flagellum. Different letters indicate significant differences (Fisher's exact test, $p < 0.05$). D: Response strength to 0.1 M sucrose of the GRNs along the flagellum. Different letters indicate significant differences (Mann-Whitney tests, $p < 0.05$). E: Example of responses and spike analyses of one GRN to inositol. The upper trace shows spikes elicited by the water responsive GRN (no response to inositol), and the spike analyses show that this is a different GRN than the small amplitude GRN responding to inositol. F: Dose-response curve of average firing to inositol. G: Distribution of inositol-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $p > 0.05$). H: Response strength to 0.1 M inositol of the GRNs along the flagellum. Different letters indicate significant differences (Mann-Whitney tests, $p < 0.05$).

Fig 3: Response properties of GRNs in *s. chaetica* of *H. virescens* to the two inorganic salts KCl and NaCl. A: Example of responses and spike analyses of one GRN to KCl. The GRN responding to KCl had small amplitude. B: Dose-response curve of average firing to KCl. C: Distribution of KCl-responding GRNs along the flagellum. Different letters indicate significant differences (Fisher's exact test, $p < 0.05$). D: Response strength to 0.1 M KCl of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann-Whitney tests, $p > 0.05$). E: Example of responses and spike analyses of one GRN to NaCl. The spike amplitude of the NaCl-responding GRN was small, whereas the additional cell firing at 0.1 M and had high amplitude, and was not considered a real response. F: Dose-response curve of average firing to NaCl. G: Distribution of NaCl-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $p > 0.05$). H: Response strength to 0.1 M NaCl of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann-Whitney tests, $p > 0.05$).

Fig 4: Response properties of GRNs in *s. chaetica* of *H. virescens* responding to ethanol and water. A: Example of response and spike analyses of one GRN to ethanol. The response to the two lower concentrations showed smaller spike amplitudes than to the highest concentration. B: Dose-response curve of average firing to ethanol. C: Distribution of ethanol-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $p > 0.05$). D: Response strength to 20 % ethanol of the GRNs along the flagellum. Different letters indicate significant differences (Mann-Whitney tests, $p < 0.05$). E: Example of a water-responsive GRN and spike analyses during stimulation with 0.0001, 0.001, 0.01 and 0.1 M sinigrin. There was no excitatory response to sinigrin, but the water-responsive GRN was inhibited with increasing concentrations of sinigrin.

Fig 5: Response properties of two different *s. chaetica* of *H. virescens* to the highest concentrations of various substances. A: Responses and spike analyses of GRNs on annulus 72 to KCl, sucrose, inositol, NaCl, sinigrin, and ethanol. (The response properties of this sensillum to three concentrations of sinigrin are shown in figure 1G). The spike shape of the sucrose-responsive GRN was broader then the other GRNs. KCl, NaCl and sinigrin might be detected by the same small amplitude GRN, whereas ethanol seemed to be detected by a large amplitude GRN. B: Responses and spike analyses of GRNs on annulus 60 to KCl, sucrose, inositol, NaCl, sinigrin, and quinine. (The response properties of this sensillum to two concentrations of quinine are shown in the two upper traces of figure 1A). Again, broad

shaped spikes of one GRN were elicited by sucrose. One GRN seemed to respond to the two salts. There was no response to sinigrin, and two different GRNs seemed to be responding to quinine and inositol.

Fig 6: Responses to sucrose and mixtures of sucrose and bitter stimuli. A: Response properties when stimulating one *s. chaetica* of *H. virescens* with sucrose and mixtures of sucrose and quinine. There was a mutual inhibition of the quinine- and sucrose-responsive GRNs. B: Average responses (imp/ s) of 92 sensilla elicited by sucrose and mixtures of sucrose and quinine, showing inhibition of the sucrose-responding GRN by quinine. C: The percentual change from the initial stimulation with sucrose, when stimulating with the quinine mixtures and the final stimulation with sucrose. D: Response properties when stimulating one *s. chaetica* with sucrose and mixtures of sucrose and sinigrin. Sinigrin inhibited the sucrose-responsive GRN. E: Average responses (imp/ s) of 44 sensilla elicited by sucrose and mixtures of sucrose and sinigrin, showing that sinigrin inhibited the sucrose-responsive GRN. F: The percentual change from the initial stimulation with sucrose, when stimulating with the sinigrin mixtures and the final stimulation with sucrose. S: 0.01 M sucrose, Q 1: 0.00001 M quinine, Q 2: 0.001 M quinine, Sin 1: 0.01 M sinigrin, Sin 2: 0.1 M sinigrin.

Table 1: Average GRN responses to two concentrations (0.001 M and 0.1 M) of sucrose, sinigrin, KCl, inositol, and NaCl, in addition to 1 M and 4.3 M ethanol and 0.00001 M and 0.001 M quinine. The percentage of the GRNs with a threshold of 0.001 M solution (1 M for ethanol and 0.00001 M for quinine) for the 7 substances is also shown.

Substance	0.001 M (imp/ s)	0.1 M (imp/ s)	% GRNs responding to 0.001 M
Sucrose	18.8	66.8	100
Sinigrin	0.5	21.2	17
Inositol	0.4	16.3	31
KCl	1.0	15.8	17
NaCl	0.3	7.7	10
Ethanol	6.6 to 5 % (1 M)	18.6 to 20 % (4.3 M)	93 to 5 % (1 M)
Quinine	2.9 to 0.00001 M	18.2 to 0.001 M	

Table 2: Response properties of 76 *s. chaetica*, allowing comparison of the responses to different substances by individual sensilla. Firing in a dose-response manner was considered as response (+).

Individual moth	*S. chaetica* of annulus #	KCl	Sucrose	Inositol	NaCl	Sinigrin	Quinine	Ethanol
1	80		+					
	77		+				+	
	76		+		+		+	
	75	+	+					
	74	+	+		+	+		
	73		+	+	+	+		+
	72		+			+	+	
	71					+	+	+
	70		+		+	+		+
	69		+	+	+		+	
	68				+			
2	58		+		+		+	
	57		+				+	
	56	+					+	
	55		+		+	+	+	
	54		+		+	+	+	
	53		+	+		+	+	
	52		+			+		+
	51		+		+	+	+	+
	50		+	+		+	+	
	49		+			+	+	
	48	+	+			+	+	
	47		+			+	+	
3	36		+				+	+
	35		+				+	
	34				+		+	
	32			+		+	+	
	31		+	+			+	+
4	31		+					
	29			+		+	+	
	28						+	
	27			+				
	26	+					+	
	24					+		+
	23			+		+		+
	22			+	+		+	+
	21	+						
	20				+		+	
	19					+	+	
	18						+	
5	36		+					+
	35	+	+			+		
	34	+	+		+	+	+	
	33		+					

	32							+
	31	+					+	+
	30	+	+			+	+	+
	29						+	
	28	+		+	+		+	
	27	+		+	+	+	+	
	26		+	+		+	+	
	25	+		+		+	+	
	24	+		+		+	+	
6	60	+	+	+		+	+	
	59	+	+		+	+	+	+
	58		+	+	+	+	+	
	57		+		+		+	+
	56	+	+		+		+	
	55		+		+		+	
	54	+	+				+	+
	53		+			+	+	
	52	+	+		+		+	
	51	+	+	+	+		+	+
	50	+	+		+		+	
	49	+	+		+	+		+
	47	+	+		+	+	+	
	46		+		+	+	+	
7	18	+			+			
	17	+				+	+	
	16	+	+			+	+	
	15	+	+	+			+	+
	14	+				+	+	+
	13	+		+			+	
	12	+				+	+	
	11		+				+	
	10						+	

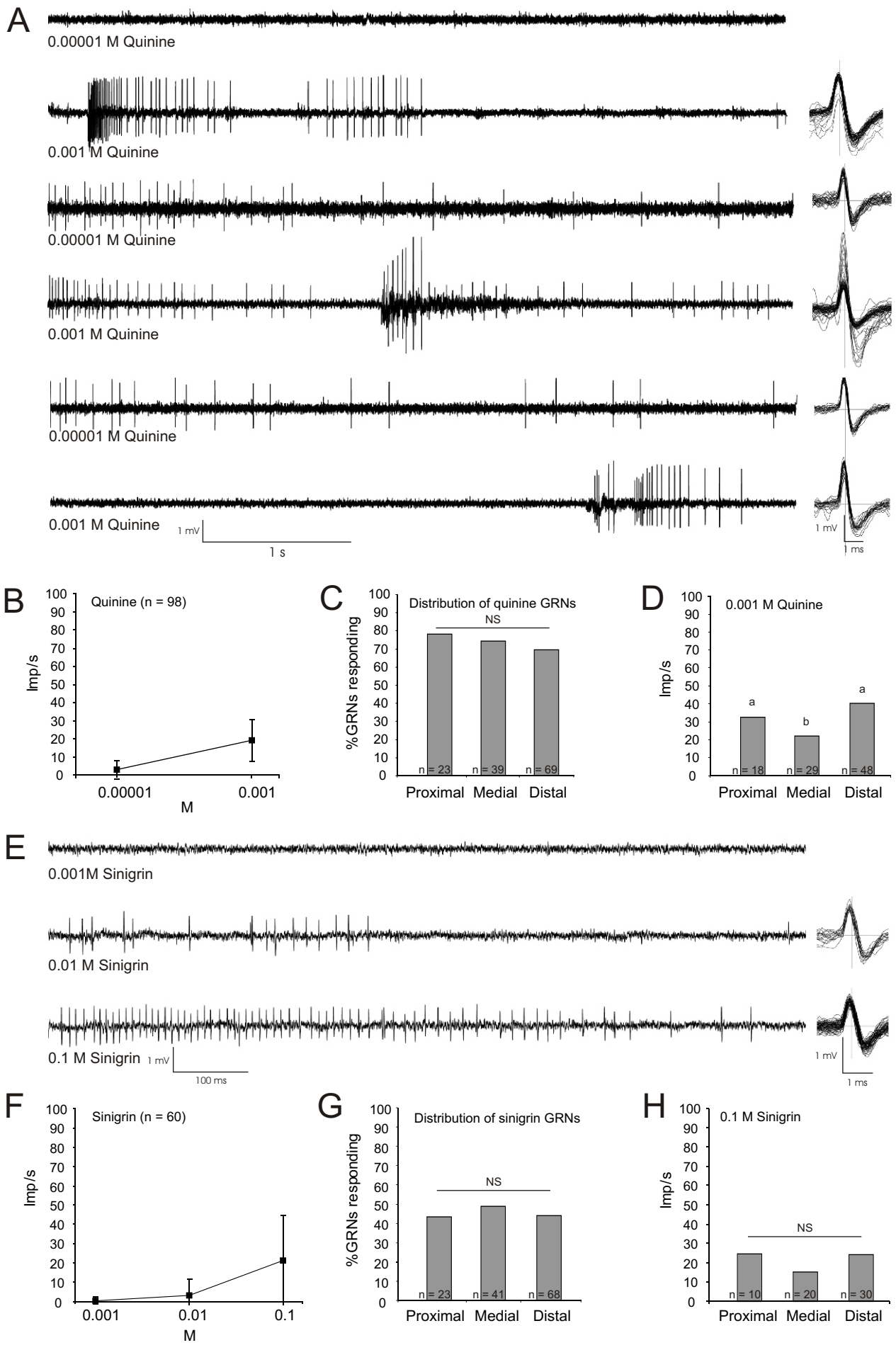
A
0.00001 M Quinine
0.001 M Quinine
0.00001 M Quinine
0.001 M Quinine
0.00001 M Quinine
0.001 M Quinine
1 mV
1 s
1 mV
1 ms
B
Quinine (n = 98)
Imp/s
M
0.00001
0.001
C
Distribution of quinine GRNs
NS
%GRNs responding
n = 23
n = 39
n = 69
Proximal
Medial
Distal
D
0.001 M Quinine
Imp/s
a
b
a
n = 18
n = 29
n = 48
Proximal
Medial
Distal
E
0.001M Sinigrin
0.01 M Sinigrin
0.1 M Sinigrin
1 mV
100 ms
1 mV
1 ms
F
Sinigrin (n = 60)
Imp/s
M
0.001
0.01
0.1
G
Distribution of sinigrin GRNs
NS
%GRNs responding
n = 23
n = 41
n = 68
Proximal
Medial
Distal
H
0.1 M Sinigrin
Imp/s
NS
n = 10
n = 20
n = 30
Proximal
Medial
Distal

Figure 1

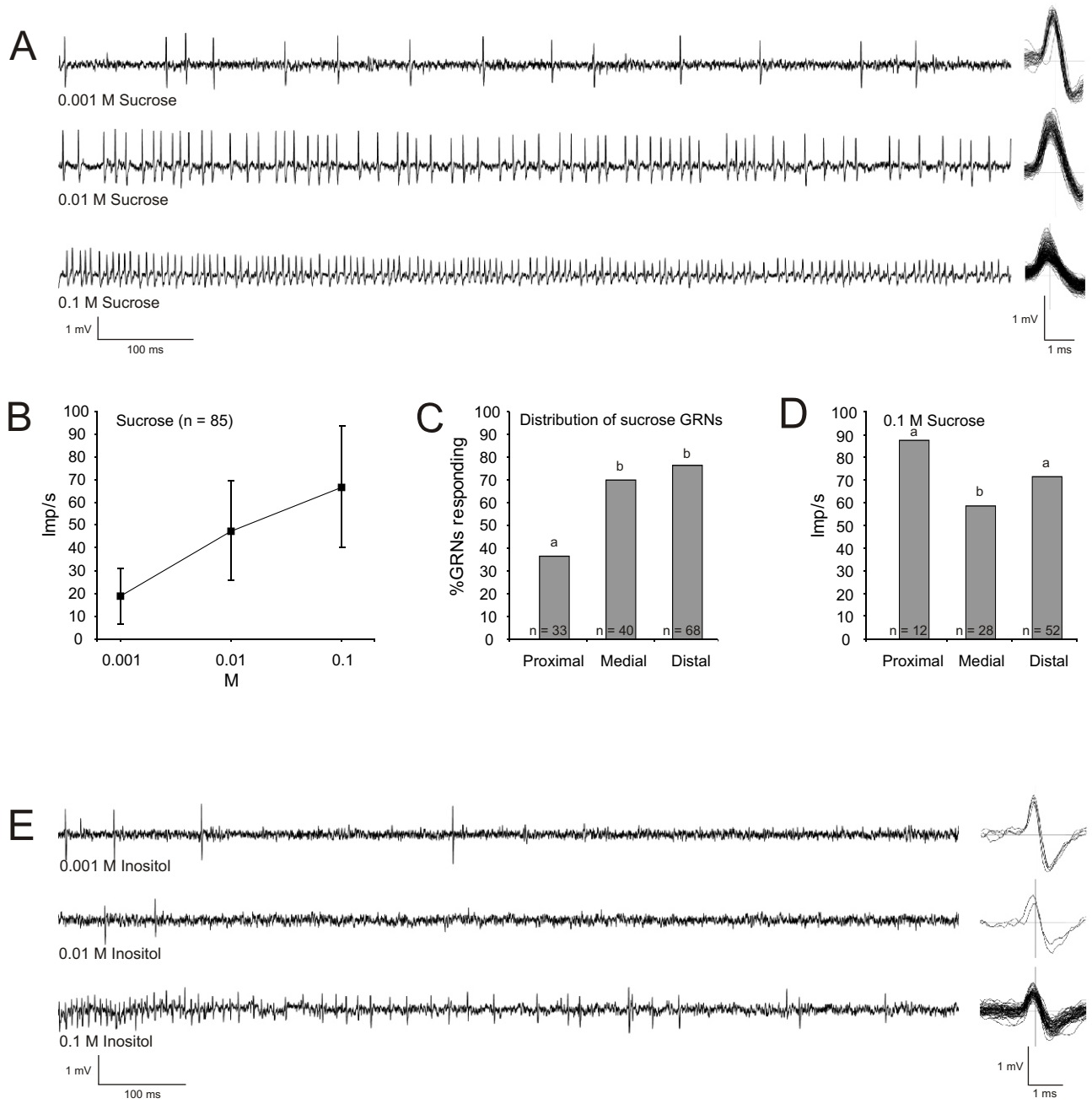
A
0.001 M Sucrose
0.01 M Sucrose
0.1 M Sucrose
1 mV
100 ms
1 mV
1 ms
B
Sucrose (n = 85)
Imp/s
M
0.001
0.01
0.1
C
Distribution of sucrose GRNs
%GRNs responding
a
b
b
n = 33
n = 40
n = 68
Proximal
Medial
Distal
D
0.1 M Sucrose
Imp/s
a
b
a
n = 12
n = 28
n = 52
Proximal
Medial
Distal
E
0.001 M Inositol
0.01 M Inositol
0.1 M Inositol
1 mV
100 ms
1 mV
1 ms
F
Inositol (n = 32)
Imp/s
M
0.001
0.01
0.1
G
Distribution of inositiol GRNs
%GRNs responding
NS
n = 23
n = 38
n = 67
Proximal
Medial
Distal
H
0.1 M Inositol
Imp/s
a
ab
b
n = 8
n = 9
n = 16
Proximal
Medial
Distal

Figure 2

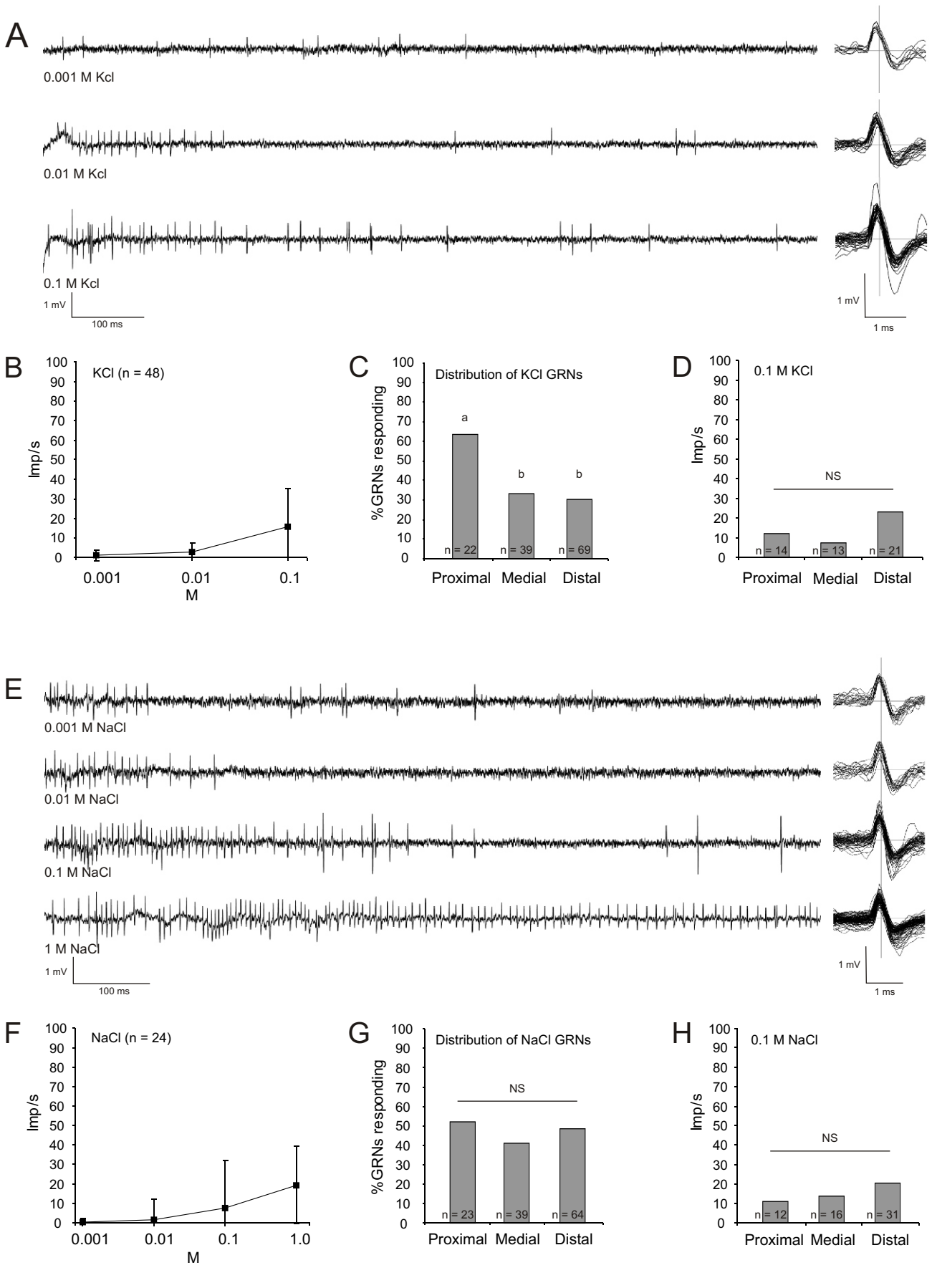
A
0.001 M Kcl
0.01 M Kcl
0.1 M Kcl
1 mV
100 ms
1 mV
1 ms
B
KCl (n = 48)
Imp/s
M
0.001
0.01
0.1
C
Distribution of KCl GRNs
%GRNs responding
a
b
b
n = 22
n = 39
n = 69
Proximal
Medial
Distal
D
0.1 M KCl
Imp/s
NS
n = 14
n = 13
n = 21
Proximal
Medial
Distal
E
0.001 M NaCl
0.01 M NaCl
0.1 M NaCl
1 M NaCl
1 mV
100 ms
1 mV
1 ms
F
NaCl (n = 24)
Imp/s
M
0.001
0.01
0.1
1.0
G
Distribution of NaCl GRNs
%GRNs responding
NS
n = 23
n = 39
n = 64
Proximal
Medial
Distal
H
0.1 M NaCl
Imp/s
NS
n = 12
n = 16
n = 31
Proximal
Medial
Distal

Figure 3

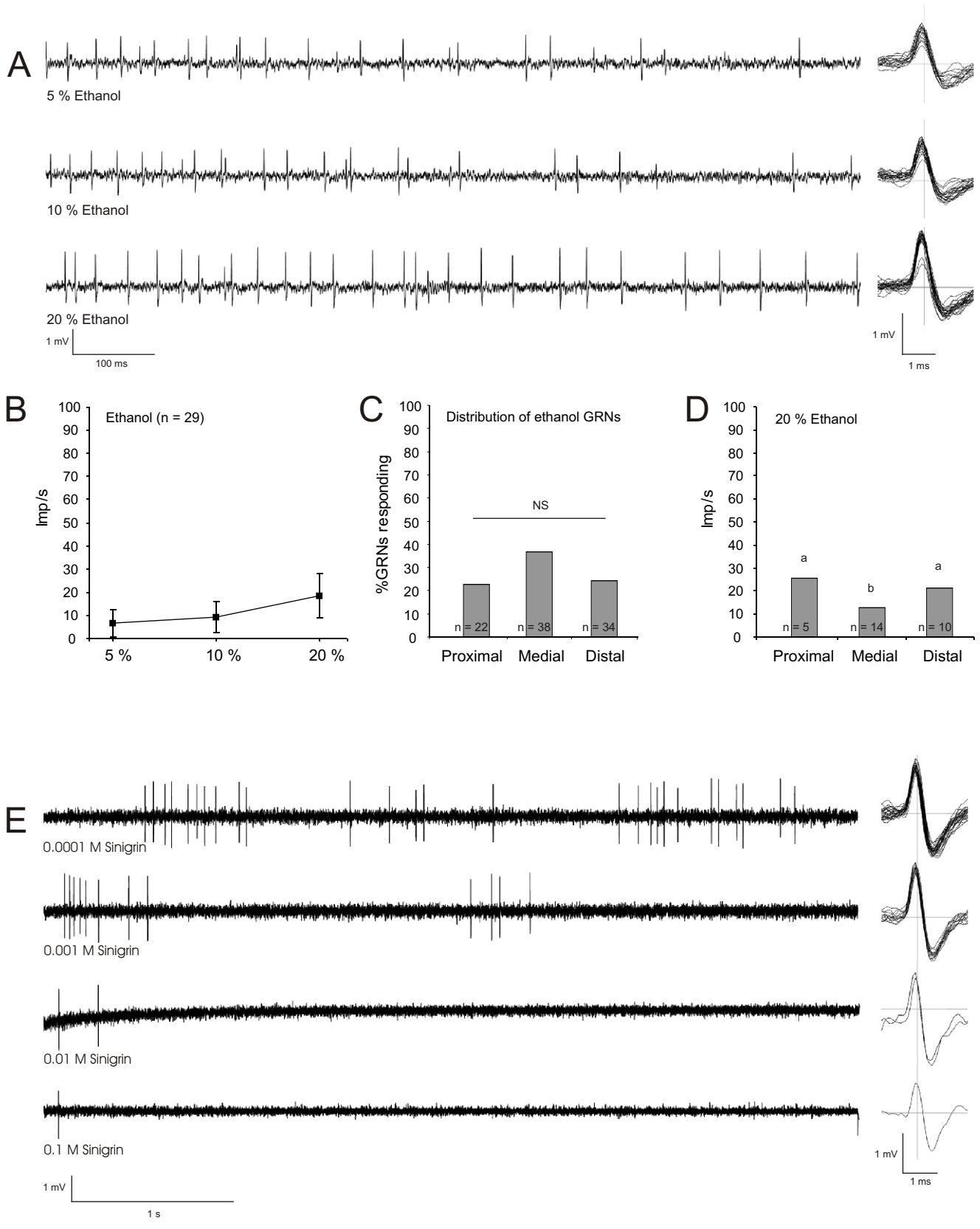
A
5 % Ethanol
10 % Ethanol
20 % Ethanol
1 mV
100 ms
1 mV
1 ms
B
Ethanol (n = 29)
Imp/s
5 %
10 %
20 %
C
Distribution of ethanol GRNs
%GRNs responding
NS
n = 22
n = 38
n = 34
Proximal
Medial
Distal
D
20 % Ethanol
Imp/s
a
b
a
n = 5
n = 14
n = 10
Proximal
Medial
Distal
E
0.0001 M Sinigrin
0.001 M Sinigrin
0.01 M Sinigrin
0.1 M Sinigrin
1 mV
1 s
1 mV
1 ms

Figure 4

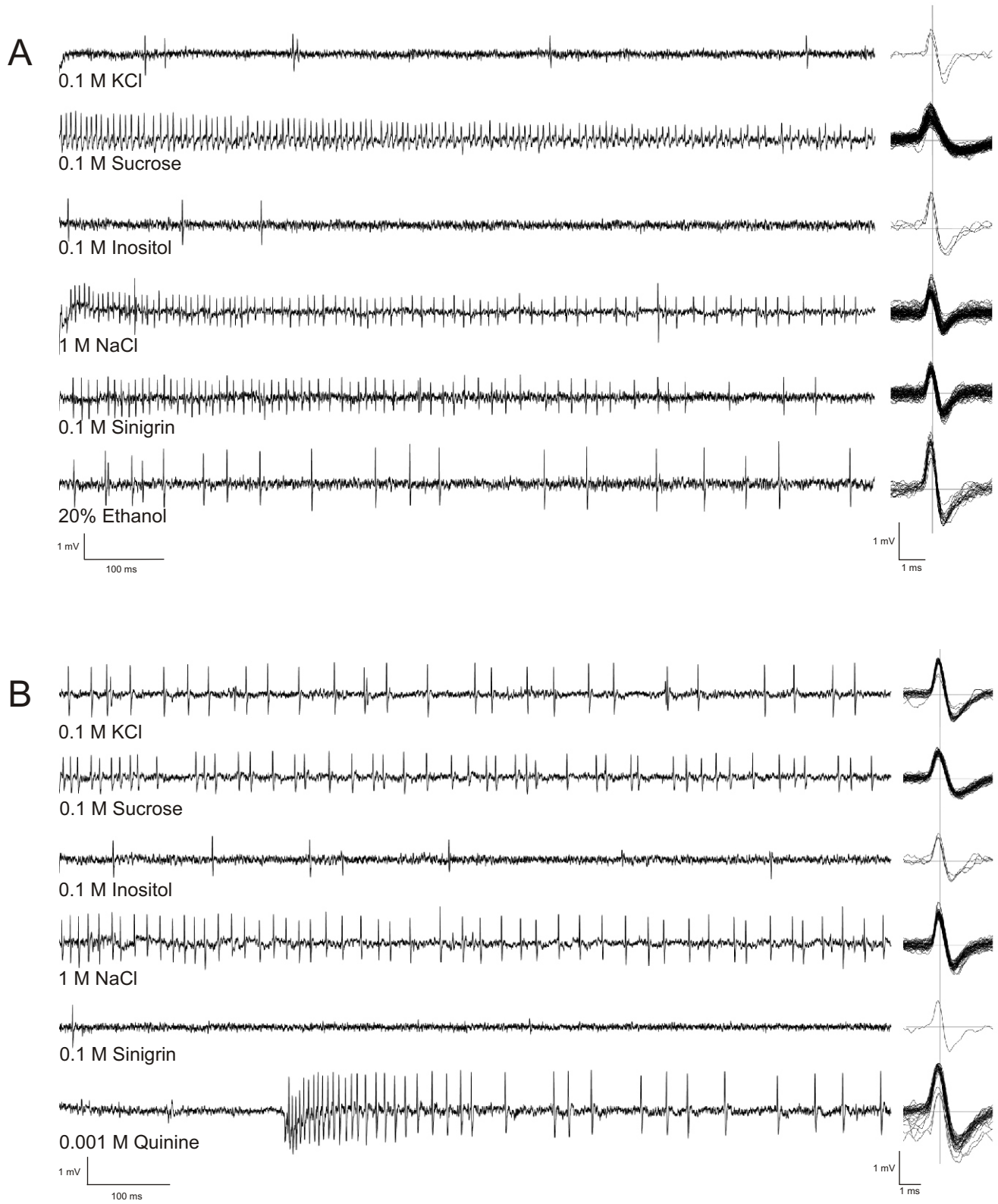
A
0.1 M KCl
0.1 M Sucrose
0.1 M Inositol
1 M NaCl
0.1 M Sinigrin
20% Ethanol
1 mV
100 ms
1 mV
1 ms
B
0.1 M KCl
0.1 M Sucrose
0.1 M Inositol
1 M NaCl
0.1 M Sinigrin
0.001 M Quinine
1 mV
100 ms
1 mV
1 ms

Figure 5

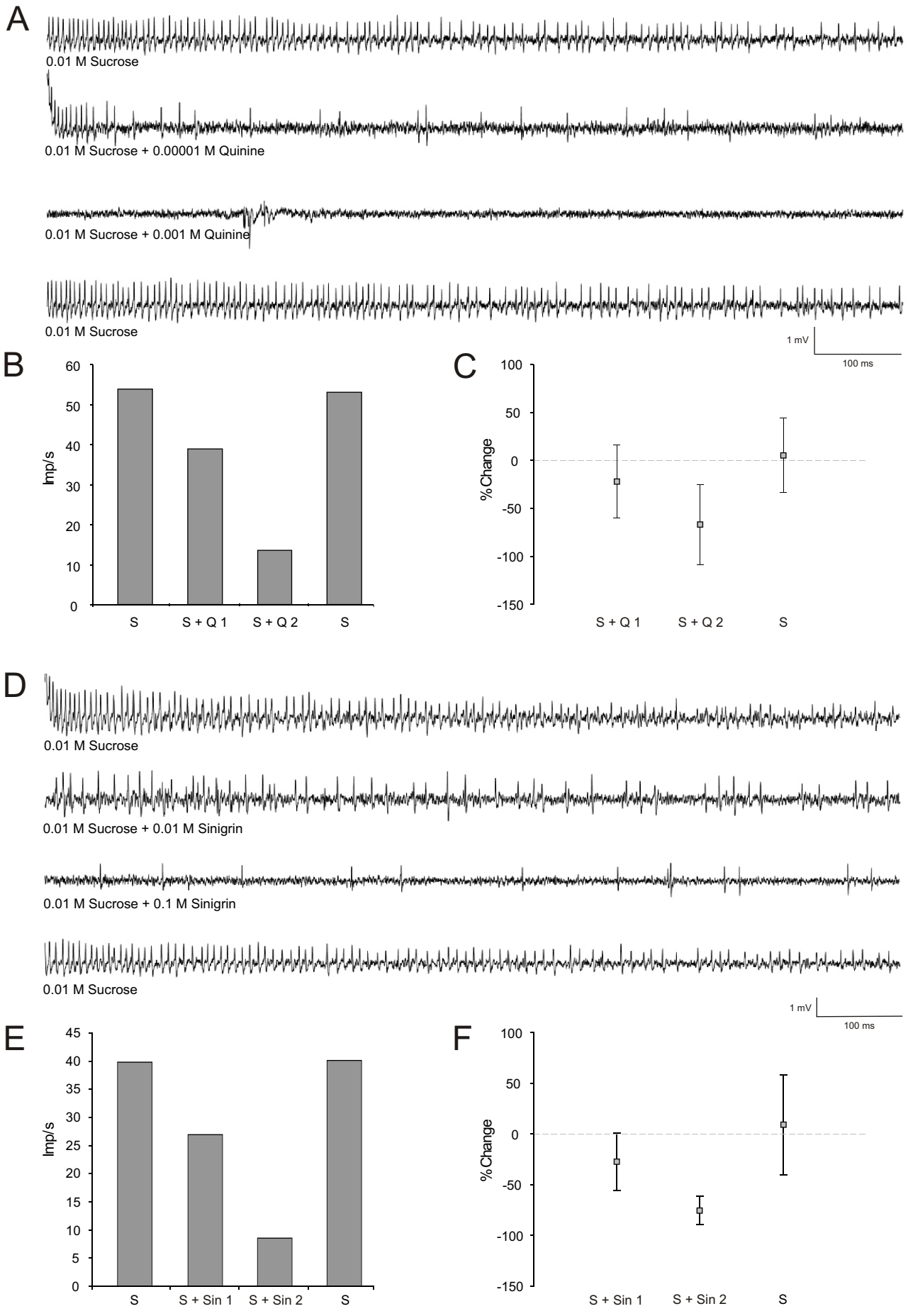
A
0.01 M Sucrose
0.01 M Sucrose + 0.00001 M Quinine
0.01 M Sucrose + 0.001 M Quinine
0.01 M Sucrose
1 mV
100 ms
B
Imp/s
60
50
40
30
20
10
0
S
S + Q 1
S + Q 2
S
C
% Change
100
50
0
-50
-100
-150
S + Q 1
S + Q 2
S
D
0.01 M Sucrose
0.01 M Sucrose + 0.01 M Sinigrin
0.01 M Sucrose + 0.1 M Sinigrin
0.01 M Sucrose
1 mV
100 ms
E
Imp/s
45
40
35
30
25
20
15
10
5
0
S
S + Sin 1
S + Sin 2
S
F
% Change
100
50
0
-50
-100
-150
S + Sin 1
S + Sin 2
S

Figure 6

Paper III

Effects of two bitter substances on olfactory conditioning in the moth *Heliothis virescens*

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Number of figures: 5

Abstract

In nature, moths encounter nutritious and toxic substances in plants, and thus have to discriminate between a diversity of tastants. Whereas olfactory learning allowing memory of nutritious plants, is well demonstrated, little is known about learning and memory of toxic items in adult lepidopterans. Moths may use bitter substances to detect and possibly learn to avoid noxious plants. We have studied the physiological and behavioural effects of two bitter substances, quinine and sinigrin, on the moth *Heliothis virescens*. Electrophysiological recordings showed responses to both compounds in gustatory receptor neurons on the antennae. The response patterns suggested a peripheral discrimination between quinine and sinigrin. We evaluated their putative aversive effect in an appetitive conditioning context where the moths learned to associate an odour with sucrose. We first aimed at enhancing olfactory conditioning of the proboscis extension response by testing the effect of the sucrose concentration on acquisition, retention and extinction. 2 M and 3 M sucrose concentration gave similar acquisition, retention and extinction performances. Experiments involving pre-exposure or facilitated extinction with an odour paired with quinine, sinigrin or no tastant showed a latent inhibitory effect, as well as an aversive effect of quinine and to a lower extent, of sinigrin. The results suggested that the two tastants may act as negative reinforcers in *H. virescens*.

Introduction

The ability to learn, remember and forget is important for the adaptation of an organism to a changing environment. In food consumption, learning and memory of the taste and smell of nutritious or noxious food is crucial for survival. For example, insects searching for nectar learn to prefer the odour of the favourable flowers. Stimulation with sucrose of the gustatory receptor neurons (GRNs) of contact chemosensilla (insect taste organs) located on different appendages of the insect body, e.g. antennae, mouthparts, and tarsi, causes the hungry insect to extend its proboscis in order to feed. This response, the proboscis extension response (PER), has been utilized to study classical conditioning, particularly appetitive olfactory learning in several insect species, including the honeybee *Apis mellifera* (Bitterman et al., 1983; Menzel, 1993; Hammer and Menzel, 1995), the bumblebee *Bombus terrestris* (Laloi et al., 1999), and several moth species (Hartlieb, 1996; Fan et al., 1997; Daly et al., 2004; Skiri et al., 2005). In all these species, including moths, it was demonstrated that the olfactory

conditioning of the PER is associative. If an initially neutral odour puff (the conditioned stimulus, CS) is given a few seconds before the sucrose stimulation (the unconditioned stimulus, US), the insects learn to associate the odour with the sucrose reward, and the CS will then trigger a conditioned response (CR), the insects extending the proboscis to the odour. In heliothine moths, previous studies have shown that they will learn to associate odours with an appetitive reward, both in the laboratory and in the field (Cunningham et al., 1999; Hartlieb et al., 1999; Skiri et al., 2005; Cunningham et al., 2006). The olfactory pathways involved in olfactory conditioning have been extensively studied and are well described in several species, including *A. mellifera* and the moth *Heliothis virescens*. The odorants are detected by olfactory receptor neurons located on the antennae, and olfactory information is transmitted via synapses within the glomeruli of the antennal lobes to local interneurons which carry out local computation, and to projection neurons (Menzel and Giurfa, 2001; Mustaparta and Stranden, 2005; Rø et al., 2007). Projection neurons further convey odour information via the antennocerebral tracts to the calyces of the mushroom bodies and to the lateral horn, a premotor area.

In the gustatory system, the sucrose solution used as US is detected by the GRNs on the antennae and the proboscis, and information is conveyed to the suboesophageal ganglion and the tritocerebrum (Mitchell et al., 1999; Kvello et al., 2006; Jørgensen et al., 2006). In *A. mellifera*, the suboesophageal-calycal tract is comprised of neurons passing on information directly from the suboesophageal ganglion to a particular area of the calyces of the mushroom bodies that is segregated from the olfactory areas (Schröter and Menzel, 2003). In addition, the ventral unpaired median neuron of the maxillary neuromere 1, VUM_{mx1}, has dendrites converging with the gustatory pathways in the dorsal suboesophageal ganglion and the tritocerebrum and axonal arborisations that converge with the olfactory pathways in the antennal lobes, the mushroom bodies and the lateral horn (Hammer, 1993). The VUM_{mx1} forms a modulatory connection between the pathways of the conditioned olfactory stimulus and the unconditioned sucrose stimulus. Electrical stimulation of this neuron in association with an odour puff is sufficient to replace sucrose reinforcement (although it does not elicit PER), suggesting that it comprises the neural substrate for sucrose reinforcement in bees. Changes of odour responses in the antennal lobes and the mushroom bodies after olfactory conditioning have been demonstrated in several studies with optical or intracellular recordings (Faber et al., 1999; Faber and Menzel, 2001; Sandoz et al., 2003; Daly et al., 2004; Yu et al., 2004).

Bitter taste, warning against the ingestion of unfavourable food is important in all organisms. Bitter stimuli constitute the largest and structurally most diverse class of gustatory stimuli, and a wide range of molecules of varying sizes and functional groups are perceived as bitter tasting (Rouseff, 1990). Both in insects and mammals, bitter taste stimuli are detected by many divergent bitter receptor proteins expressed in single GRNs (Adler et al., 2000; Thorne et al., 2004; Wang et al., 2004; Mueller et al., 2005). In the fruitfly *Drosophila*, the receptor proteins are co-expressed in subsets of bitter GRNs. If the different subsets of bitter GRNs synapse on different interneurons or motorneurons in the CNS, or if several transduction mechanisms are involved, passing on different information to the downstream neurons, this would provide mechanisms enabling flies to discriminate between bitter tastants. In insects, different bitter stimuli may elicit different behavioural reactions, indicating the presence of a differential coding system (Glendinning and Hills, 1997).

In the present study, two bitter substances that are indiscernible to humans were tested for their aversive value in *H. virescens*. The prototypical bitter compound, quinine, is an alkaloid known to act through blocking of certain K^+ channels in vertebrates or permeate cell membranes directly and activate G-proteins, bypassing the receptor in *in vitro* preparations (Spielman et al., 1992; Naim et al., 1994). We also chose sinigrin (a glucosinolate) because it was previously found to be non-appetitive in *H. virescens* (Blaney and Simmonds, 1988; Jørgensen et al., 2006). Analyses of antennal GRN responses to the two substances were performed and their aversive effects were tested in the appetitive context of olfactory conditioning of PER. Two main protocols were used to study the aversive effect of the two tastants. In the first protocol (pre-exposure), moths were pre-exposed to the odour CS associated to one of the tastants (no tastant as control), and the success of subsequent acquisition of the same CS and sucrose was observed. In the second protocol (facilitated extinction), moths were first subjected to an acquisition phase with CS and sucrose, before being subjected to an extinction phase, where the same CS was associated with one of the tastants (no tastant as control). Possible facilitation of extinction was determined. Such experiments where a decrease in CRs is expected due to the bitter stimuli, has to rely on high learning rates. A previous study of appetitive conditioning in *H. virescens* analysed the effect of CS quality and concentrations (Skiri et al. 2005). Conditioning with increased CS concentrations increased the learning rate, and odorants activating different receptor neuron types caused different learning performances. *Racemic* linalool induced strong and reliable learning, and was chosen as CS in the present study. However, the effect of sucrose concentration on learning success was unknown. Therefore, we first performed an experiment

comparing the effect of two high sucrose concentrations (2M and 3M) on acquisition of CRs, retention between 15 min and 48 h, and resistance to extinction at the same intervals. This allowed us to choose adequate conditions for the pre-exposure and facilitated extinction experiments with the bitter substances.

Material and methods

Insects and preparation

Adult *H. virescens* (Fabricius) used in the experiments were received as pupae from Syngenta, Basel, Switzerland. The male and female pupae were sorted and hatched in separate climate chambers (22°C, reversed photoperiod, Refritherm 200, Struers-Kebolab, Albertslund, Denmark). Experiments with males and females were carried out in separate groups. Newly hatched insects were placed in containers with free access to 5 % (w/v) sucrose solution. After 24 h the insects were immobilized in Plexiglas holders with tape between the head and the thorax, exposing the head with the proboscis and the antennae. The insects were then deprived of food for 48 h in the climate chambers. One hour before the experiments started the insects were placed in the experiment room for familiarisation to the experimental context.

Test compounds

The odorant used as CS was *racemic* linalool (95 % checked in GC, Sigma-Aldrich, Steinheim, Switzerland) which was diluted in n-hexane (99 %, v/v, 1:100), and stored at minus 20°C. A dose (100µl) of this solution was applied to a piece of filter paper (160 mm diameter) from which the n-hexane evaporated before it was placed in a glass cartridge sealed with Teflon caps. Each cartridge was used for 1 h (maximum 124 stimulations), and was made the day of the experiment. The appetitive stimuli were 2 M or 3 M sucrose (99.9 %, Sigma-Aldrich). The 3 M solution was put on a stirrer for 4-5 hours at room temperature for all the sucrose to dissolve. The putative aversive stimuli were 1 M sinigrin monohydrate (99 %, VWR International, Oslo, Norway) or 0.16 M quinine hydrochloride dihydrate (98 %, VWR International). Because of the low solubility of quinine in water, this was the highest possible molarity without adding acid or alcohol. Quinine (0.01 mM, 0.1 mM) and sinigrin (1.0 mM, 10 mM, 100 mM) were solved in the electrolyte 0.01 M KCl (99,5 %, Sigma-Aldrich) for the electrophysiological recordings.

Experiment 1

US concentration, retention and extinction

The experiments were carried out in a dimly lit room with a constant temperature of 23°C. One at a time, each moth was placed in front of a ventilation outlet with a weak suction. Facing the insect at 2 cm distance was a glass tube with a constant air flow (~ 400 mL/min). The cartridge containing the CS was inserted into the tube, and the odour stimulus was given as a 5 s puff of ~100 mL/min flow into the constant air stream. The sucrose US (5 s) was applied with a toothpick 2.5 s after the onset of the odour puff, first to both antennae, and then to the extended proboscis. Since moths tend to be unresponsive at the beginning of conditioning due to low attention, the same method as in previous work was used to ensure learning success (Skiri et al., 2005): If the insect did not extend its proboscis at first encounter with the sucrose, the proboscis was forced out, and the insect was allowed to drink. This was not done in subsequent trials, meaning that the insects that failed to show PER were not rewarded. Each insect was placed in the setup 15 s before CS onset in order to adapt to the air flow, and was removed 10 s after the end of the US. For each insect there were 8 conditioning trials with 15 min inter-trial intervals (ITI). Subsequently there were 8 extinction trials where the odour was given without reward (15 min ITI). At the end of every experiment, all insects were tested for the unconditioned response (UR) to sucrose. The results were calculated as the percentage of insects that showed CR during each stage of the conditioning trials and the extinction trials. To find out if US concentration affected acquisition, retention, or extinction, 2 M and 3 M concentrations were used as US in conditioning experiments in different insects. Each of the 2 groups were further divided into 5 retention groups, for which the first extinction trial started after the last acquisition trial at 15 min, 2 h, 8 h, 24 h, or 48 h, respectively. All retention periods were tested in each experiment. The different parameters were chosen according to previous conditioning experiments in *H. virescens* (Skiri et al., 2005).

Experiment 2

Antennal gustatory neuron responses to quinine and sinigrin

Electrophysiological recordings from GRNs of *sensilla chaetica* on the *H. virescens* antennae were carried out using a tip recording technique (Hodgson et al., 1955). The recording electrode (thin walled borosilicate glass capillaries, Harvard apparatus, UK) was pulled in a 2-step electrode puller (PP-830, Narishige group, Japan) to a tip diameter of approximately 10-20 µm. To avoid crystallisation and concentration changes at the tip, the electrode was filled

with the test substance just a few seconds before the start of the recording. The recording electrode containing the test solution was placed over single sensilla hairs for 5 s with an inter-stimulus interval of at least 10 min to avoid adaptation. Taste sensilla from all parts of the flagellum were included in the experiments. The recording glass electrode was connected to a TastePROBE amplifier (10x, Syntech, Hilversum, Netherlands) (Marion-Poll and Van der Peers, 1996) and the signals filtered (low pass: 50 Hz and high pass 3000 Hz) using the CyberAmp 320 from Axon Instruments (Burlingame, CA). The reference electrode was a 1 mm AgCl coated silver wire placed in the moth abdomen. Analysis of the spikes was performed using the software AutoSpike-32 (Syntech). The responses were counted as number of spikes elicited during the 5 s stimulation period, and the temporal patterns were assayed, counting spikes in 0.5 s bins.

Experiment 3

CS pre-exposure associated with putative aversive stimuli

In this experiment we tested whether the bitter compounds sinigrin and quinine could induce aversive effects on the subsequent learning of odour-sucrose associations. The experiment consisted of 2 phases, a pre-exposure phase and a conditioning phase. In the pre-exposure phase, 3 groups of insects were pre-exposed to different stimuli 8 times (15 min ITI). In the control group each insect was exposed to linalool (5 s) paired with stimulation with a dry toothpick (5 s, no tastant, mechanosensory control) of the antennae 2.5 s after the onset of the linalool stimulus. In the two bitter treatment groups the insects were exposed to linalool (5 s) paired with 1 M sinigrin or 0.16 M quinine stimulation, respectively, applied with a toothpick. Bitter tastant stimulation started 2.5 s after the onset of the linalool stimulus and lasted 5 s. Since the aversive value of the tastants might be mediated by GRNs on the proboscis as well as on the antennae, the stimulation was first applied to the antennae, and then to the proboscis. At the first trial, after antennal stimulation, the proboscis was forced out and the bitter tastant or dry toothpick was shortly applied. In nature, if the insect extends the proboscis to an antennal stimulation, it expects to taste the compound with the proboscis. This process could be necessary for choosing to accept or avoid a given food. For this reason, in subsequent trials, moths that extended the proboscis to the tastant received a stimulation of the proboscis. In our control group, moths received CS presentations without sucrose before the acquisition, which could lead to a so-called latent inhibition effect, i.e. a resistance to acquisition. To test for this effect we included a fourth untreated control group in which the moths were left without pre-exposure. In the conditioning phase (starting 15 min after the end of the pre-

exposure phase), all groups were subjected to an identical acquisition procedure, with 8 conditioning trials (CS associated to 2 M sucrose US) with 15 min ITI, as in experiment 1. After 15 min, all moths received a retention test with the CS alone for 5 s.

Experiment 4

Extinction of CR combined with putative aversive stimuli

The goal of this experiment was to evaluate the aversive effects of bitter tastants when applied during extinction. The experiment consisted of 2 phases, a conditioning phase and an extinction phase. In the conditioning phase, all insects were conditioned to linalool with 2 M sucrose (described in experiment 1). In the extinction phase (starting 15 min after the end of the conditioning phase) the insects were divided in 3 groups receiving different types of extinction trials (8 trials, 15 min ITI). The control group was given a dry toothpick (no tastant, mechanosensory control) on the antennae and on the proboscis, when extending the proboscis to the CS. The 2 treatment groups were given 1 M sinigrin or 0.16 M quinine, respectively, with a toothpick on the antennae and on the proboscis, when extending the proboscis to the CS.

Statistics

Behaviour

All insects that failed to show UR 3 times or more during acquisition or at the end of the experiment were considered unmotivated and excluded from the data analysis. To compare extinction performance independently of different retention levels, only insects showing CR at the first extinction trial were included in the analysis (Fig 1D and 5B). Comparisons of acquisition or extinction performance among groups were carried out on the sum of conditioned responses given by each moth during the respective phase, using Mann-Whitney tests (for $n = 2$ groups) or Kruskal-Wallis tests (for $n > 2$ groups). Performance at individual trials was compared between groups using Fisher's exact tests. Depending on the question addressed in each experiment, either multiple comparisons with threshold corrections (experiment 1) or planned comparisons without threshold correction (experiments 3 and 4) were performed. In experiment 1, we compared extinction at different retention times. After a global Kruskal-Wallis test, we carried out multiple comparisons using the Noether method (1976, in Scherrer 1984). The alpha level was corrected using the Dunn-Sidak threshold correction [$\alpha' = 1 - (1 - \alpha)^{1/k}$ where k is the number of two-by-two comparisons in which each data is used]. The goal of experiments 3 and 4 was to test specifically the effect of bitter

compounds in appetitive conditioning situations. Therefore, we only carried out a few planned comparisons between performance in the bitter-treated groups and the control group, using Mann-Whitney tests with an alpha-level of 0.05 (the number of planned comparisons being always lower than the number of degrees of freedom (n groups – 1) of the experiment).

Electrophysiology

To compare the time courses of responses of the receptor neurons to the different concentrations of tastants, 2-way *tastant x time bin* ANOVA were carried out (with repeated measurements). Two-by-two comparisons of tastant responses were carried out with 1-way ANOVA, using the Dunn-Sidak threshold correction as above. Comparisons between tastants at individual time bins were done using Scheffé tests for multiple comparisons.

Results

Experiment 1

Out of the 554 moths used in the experiment, 348 (62.8%) were included according to the criteria listed in the methods chapter.

Effect of sucrose concentration on acquisition

Conditioning with 2 M and 3 M sucrose as US induced good acquisition, where the responses to the odour increased with trials, from zero at the first conditioning trial (no spontaneous responses), to 50% and 45% at the eighth conditioning trial for the 2 M and 3 M groups, respectively (Fig 1A). The acquisition curves did not reach asymptotic levels after eight conditioning trials, indicating that more trials might further have enhanced the learning success. Acquisition was similar in the 2 groups (Mann-Whitney test, $z = 0.59$, $p = 0.56$).

Effects of time after training and sucrose concentration on retention

Retention time is the period between the last conditioning trial and the first extinction trial. The effect on retention of time elapsed after training was studied by comparing responses of the first extinction trial performed after 15 min, 2 h, 8 h, 24 h, and 48 h in different groups of moths (Fig 1B). Overall, memory decreased with time, being strongest at 15 min and declining gradually to a lower level at 48 h. Retention was highest in the 2 M reward group tested after 15 min where the proportion of insects responding was 67% and lowest (21%) in the 3 M reward group tested after 48 h. An exception from the gradually declining response

with time appeared for the 3 M group, showing a slightly stronger retention after 24 h than after 8 h. No statistical differences between the 2 concentrations at any of the retention times were found (Mann-Whitney, 15 min: $p = 0.473$; 2 h: $p = 1$; 8 h: $p = 0.626$; 24 h: $p = 0.311$; 48 h: $p = 1$), so the data of the 2 M and 3 M groups were pooled before testing whether the first extinction trial differs between the 5 retention groups. The 15 min and 2 h groups were significantly different from the other retention groups (Fisher's exact tests, all $p < 0.01$), but not from each other ($p = 1$). The 8 h, 24 h, and 48 h groups were not significantly different from each other (Fisher's exact tests, $p > 0.04$) when the α-level was corrected for multiple comparisons (Dunn-Sidak correction, $\alpha' = 0.0127$).

Effect of time on extinction

To compare the strength of the odour-sucrose association at different times after conditioning, we assessed its resistance to extinction during the 8 extinction trials (Fig 1C). To be able to compare extinction between groups, despite the differences observed in absolute retention scores (see above), only moths showing a CR at the first extinction trial were included (Fig 1D). In all cases the responses decreased with increasing number of extinction trials. The moths tested after 8 h showed the fastest and highest overall extinction, the percentage of responses declining to 4% at the last trial. The 48 h group showed a slower and lower overall extinction than the other groups, 40% of the moths still showing CR at the last trial. There was a significant heterogeneity in overall extinction among the 5 groups (Kruskal-Wallis, $p = 0.03$). Two-by-two comparisons indicated that extinction in the 48 h group was significantly lower than in the 8 h and the 24 h groups (Noether multiple comparisons with Dunn-Sidak correction, $z = 3.11$ and $z = 2.53$, respectively, $p < 0.0127$) and just short of significance compared to 15 min and 2 h groups ($z = 2.35$ and $z = 2.39$, respectively, $p < 0.02$). Although retention decreased with the interval between acquisition and extinction, the remaining association was strongest for the 48 h interval.

Experiment 2

Antennal gustatory neuron responses to quinine and sinigrin

When applying different concentrations of sinigrin and quinine to the contact chemosensilla, *s. chaetica*, on the flagellum of the *H. virescens* antenna, responses to the 2 substances seemed to be elicited in separate receptor neurons. A bursting firing pattern was elicited in one type of receptor neuron during stimulation with 1 mM quinine compared to no activity when stimulating with the electrolyte KCl (Fig 2A-B). The GRN responding to quinine often

showed a long latency, and the bursts appeared at varying intervals in different recordings. The same concentration of sinigrin induced only a few spikes with smaller amplitude and no bursting activity when recording from the same sensillum (Fig 2A). When increasing the concentration of sinigrin to 100 mM, the number of spikes per 5 seconds was in the same range as that of 1 mM quinine, enabling comparison of the average temporal firing patterns induced by the 2 substances (Fig 2, 3A). Sinigrin elicited a phasic-tonic firing, and quinine a bursting firing. The bursting response to quinine did not change across recordings, and was similar in sensilla showing responses to quinine alone or both quinine and sinigrin. The mean responses to quinine and sinigrin in 74 sensilla plotted in 0.5 s bins showed the temporal differences in firing patterns (Fig 3B). Because the bursts of the quinine responsive GRNs appeared at varying intervals in different recordings, the average response appeared as a sustained high level of firing throughout the 5 s. For comparison, the average temporal response patterns to 1 mM sinigrin and the electrolyte 10 mM KCl were included in the figure. There were significant differences in the average overall responses to the different tastants. A 2-factor ANOVA on the effects of *tastants* and *time bins* (both repeated measures) indicated a significant *tastant* effect ($F_{3.219} = 15.48$, $p < 0.001$), a significant *time bin* effect ($F_{9.657} = 42.76$, $p < 0.001$) and a significant interaction ($F_{27} = 12.79$, $p < 0.001$). In particular, the time courses of spiking activity were significantly different between responses to 1 mM quinine and 100 mM sinigrin (*tastant* x *time bin* ANOVA, $F_{9.657} = 10.21$, $p < 0.001$), although the average response over the 5 s to the 2 tastants was not different (*tastant* ANOVA, $F_{1.73} = 3.60$, $p = 0.06$). The responses to 10 mM KCl and 1 mM sinigrin over the 5 s were not significantly different (*tastant* ANOVA, $F_{1.73} = 0.42$, $p = 0.51$), but the response to both substances differed from the response to 100 mM sinigrin and 1 mM quinine (*tastant* ANOVA, $F_{1.73} > 8.68$, $p < 0.01$). During the first 0.5 s (*tastant* effect: $F_{3.219} = 17.00$, $p < 0.001$), the response to 100 mM sinigrin was significantly higher than that to 1 mM quinine, indicated with letters in the first dotted square in figure 3B (Scheffé test, $p = 0.004$), but by the third time bin (1-1.5 s, *tastant* effect: $F_{3.219} = 13.84$, $p < 0.001$), the relationship was reversed, the response to 1 mM quinine being significantly higher than the 100 mM sinigrin response, indicated with letters in the second dotted square in figure 3B (Scheffé test, $p = 0.0005$). A high proportion of the sensilla (93%) had GRNs responding to 1 mM quinine, whereas 83% of the sensilla had GRNs responding to 100 mM sinigrin, and 68% to the electrolyte 10 mM KCl. A few sensilla (5%) had GRNs that responded to 100 mM sinigrin, but not to 1 mM quinine, whereas 15% of the sensilla had GRNs responding to quinine, but not to sinigrin. Twenty-one percent of the sensilla had GRNs responding to quinine and

sinigrin, but not to KCl. These results suggested that sinigrin and quinine are detected by different GRNs on the moth antennae. The putative aversive effect of the 2 substances was tested in the following experiments.

Experiment 3

Out of the 338 moths used in the experiment, 230 (68%) were included according to the criteria listed in the methods chapter.

Acquisition after CS pre-exposure associated with quinine or sinigrin

During pre-exposure, no insects showed PER to the odorant linalool while 3.4% of the insects showed PER to the dry toothpick (mechanosensory control), 3.5% to quinine and 24.6% to sinigrin (Fig 4A). The quinine group did not differ from the control (Mann-Whitney test, $z = 0.052$, $p = 0.958$), whereas stimulation with sinigrin elicited significantly more PER than in the control (Mann-Whitney test, $z = 3.38$, $p = 0.001$).

Acquisition in the control group reached 25% at the end of training, while moths treated with CS + quinine reached only 11%, and moths treated with CS + sinigrin only 13% (Fig 4B). However, in untreated moths, not receiving linalool in the first phase, acquisition reached 42%. Acquisition performance was significantly lower in the quinine group compared to the control (Mann-Whitney test, $z = 2.28$, $p = 0.023$), but not in the sinigrin group (Mann-Whitney test, $z = 1.24$, $p = 0.217$). Acquisition in untreated moths was significantly higher than in the control group (Mann-Whitney test, $z = 1.94$, $p = 0.05$), meaning that pre-exposure to the CS and mechanosensory stimulus (no tastant) led to a resistance to acquisition. The treatment with quinine enhanced this effect leading to significantly higher resistance to acquisition. The differences in acquisition were not due to differences in the appetitive motivation of the moths, since no significant effects of the pre-exposure treatments on subsequent UR to sucrose in the acquisition phase appeared (Mann-Whitney test, control vs. quinine: $z = 0.247$, $p = 0.805$, control vs. sinigrin: $z = 0.838$, $p = 0.402$, control vs. untreated moths: $z = 1.532$, $p = 0.126$).

The results of a retention test 15 min after acquisition showed the same pattern of response for the bitter compounds: retention was significantly lower in the quinine group compared to the control group (Fisher's exact test, $p = 0.045$), but not in the sinigrin group (Fisher's exact test, $p = 0.21$). However, retention in untreated moths was not significantly higher than in controls (Fisher's exact test, $p = 0.121$).

This experiment shows a putative aversive effect of quinine on subsequent acquisition. Although sinigrin gave similar results as quinine, no significant difference was found in acquisition between control and sinigrin-treated moths. This experiment also shows that pre-exposure with the CS (here with a mechanosensory stimulation) reduces subsequent acquisition of the CS-sucrose association. This effect suggests the possible existence of a latent inhibition phenomenon in moths. In the following experiment we addressed the putative aversive effects of quinine and sinigrin in a different learning situation.

Experiment 4

Out of the 398 moths used in the experiment, 294 (73.9 %) were included according to the criteria listed in the methods chapter.

Facilitated extinction of CR combined with quinine or sinigrin

Acquisition was efficient in all groups, reaching 32-34% at the end of training, without any significant difference between treatment and control groups (Fig 5A, Mann-Whitney, quinine vs. control, $z = 0.299$, $p = 0.77$, sinigrin vs. control, $z = 0.568$, $p = 0.57$). 39-43% of the moths showed CR in the first extinction trial. To compare extinction on an identical basis in the different groups, only these insects were included (Fig 5B). Extinction was strong in all groups, responses declining with repeated trials, down to 17% in the control group, and 0% and 2% in the quinine- and sinigrin-treated groups, respectively (Fig 5B). Extinction was significantly stronger both in the quinine group (Mann-Whitney, $z = 2.5$, $p = 0.012$) and in the sinigrin group compared to the control group (Mann-Whitney, $z = 2.12$, $p = 0.03$).

Discussion

The first part of this study (Fig 1) was aimed at improving the PER conditioning protocol previously used in heliothine moths (Skiri et al., 2005), as well as investigating the duration of the established memory and the resistance of the CS-US association to contradictory information. All these parameters were crucial for assessing the aversive effects of bitter stimuli. We found similar learning performances when using 2 and 3 M sucrose as rewards. However, in a previous study with the same CS and 1 M sucrose reinforcement (Skiri et al., 2005), we obtained only 29% CR in the last trial, compared to 45-50% obtained with 2 M and 3 M sucrose in the present study. This observation shows that the strength of the US may be important for acquisition in *H. virescens*, as is generally observed in learning studies. The

same observation was made in other insects, like the honeybee and the bumblebee (Bitterman et al., 1983; Loo and Bitterman, 1992; Laloi et al., 1999; Scheiner et al., 1999; Scheiner et al., 2004). In moths, a saturation of the reinforcing effect of sucrose seems to be reached with 2 M sucrose solution.

Eight spaced conditioning trials were sufficient for the moths to remember the CS-US association for at least 48 h. This implies that moths, although non-social insects with an adult life span of approximately two weeks, can build long memories. In comparison, *A. mellifera* receiving three spaced appetitive learning trials will remember the odour for the rest of their lives (several weeks) (Sandoz et al., 1995; Menzel, 1999), *Drosophila* remember odour-electric shock associations for seven days after 10 spaced aversive conditioning trials (Tully et al., 1994), and memory after four-trial differential conditioning in the crickets lasts one week (Matsumoto and Mizunami, 2002).

The moths tested after 15 min and 2 h showed the highest retention performances. The responses dropped to a lower level after 8 h, suggesting that it is most important for moths to remember an odour within a few hours, and probably less important to remember it for several hours or days. In contrast to honeybees, learning of plant odorants in moths serves only self consumption and oviposition purposes. A strong memory shortly after learning may therefore be well adapted to the life of the moth. It is possible that the 15 min and 2 h memories constitute the same forms of memory in the moth, both because of equally high retention and equal resistance to extinction in the two groups, suggesting similar consolidation statuses at the two time intervals. These memories in the moths could be equivalent to the late short-term memory phase described in honeybees, developing over time in the minute range, and used to remember rewards (nectar quality and quantity) between flower patches (Menzel, 1999). In honeybees, this memory stage is transient, and sensitive to retrograde amnesia or additional experience (Erber, 1976; Menzel, 1990). Memory then consolidates to a more stable and amnesia resistant middle term memory within approximately 1 h (Menzel, 1990). In *Drosophila* as well, memory is sensitive to cold treatment in the first hour after conditioning (Tully et al., 1994). Experiments using cold treatment after conditioning in moths may help examine amnesia-sensitive and amnesia resistant memories, providing further insights into memory phases underlying performance. In contrast to honeybees, retention after two hours in the moths declined quickly with time, and was lowest in the group tested after 48 h. In this group, there was a strong resistance to extinction, suggesting that the CS-US association was strong and stable in the moths that remembered the odour. Two different types of stable long-term memory have been described in other insects; one corresponds to the

early long-term memory found in honeybees as well as the anaesthesia-resistant memory in *Drosophila*, that are both resistant forms of memory, independent of protein synthesis (Wittstock et al., 1993; Tully et al., 1994; Wüstenberg et al., 1998). The second type is the protein synthesis (transcription) dependent late long-term memory that is found as early as 5 h after conditioning in crickets (Matsumoto et al., 2003) or as late as 3-4 days in honeybees. Future experiments using protein synthesis inhibitors will reveal which memory phase controls 48 h retention in moths.

The presented electrophysiological recordings show excitatory responses to both quinine and sinigrin in GRNs on the moth antennae. In contrast, one study of the honeybee antennae showed no excitatory responses of GRNs to the bitter substances tested (De Brito Sanchez et al., 2005). In our study, sinigrin and quinine might be detected by two different GRNs (Fig 2-3). This assumption is based on the different temporal firing patterns elicited when stimulating with the two tastants. The bursting firing pattern of the GRNs responding to quinine differs significantly from the phasic-tonic firing pattern elicited in the GRNs responding to sinigrin. Some classes of bitter substances, like quinine, are known to elicit a bursting firing pattern in GRNs whereas others are not (Dethier, 1976; Chapman et al., 1991). The observed differences in firing pattern in the present recordings was not due to differences in response intensity, since the temporal firing pattern for sinigrin did not change when the concentration was increased to elicit the same number of spikes as quinine. Moreover, the few sensilla with neurons responding to sinigrin, but not to quinine and vice versa, further support the assumption of two separate GRNs mediating information about the two tastants. An alternative explanation is that one GRN might respond to both substances, eliciting different temporal firing patterns, where two different receptor types and possibly different excitatory transduction pathways are involved, as suggested in the tobacco hawkmoth *Manduca sexta* larvae (Glendinning and Hills, 1997). Having several receptor proteins for different bitter substances in the same GRN would increase the chances of the insects to detect the components in mixtures of bitter plant substances that are potentially toxic or nutritious. An important presumption for the discrimination mechanism in this case would be that the CNS could differentiate the different spike firing patterns of the same GRNs. Regardless of whether there are one or two GRN types for sinigrin and quinine, our results suggest that the gustatory system of moths is able to discriminate between these two substances.

The putative aversive effects of the two substances were elucidated using pre-exposure (Fig 4) and facilitated extinction experiments (Fig 5). In the pre-exposure experiments, only quinine was shown to be significantly aversive, although a clear tendency appeared for

sinigrin as well. In the facilitated extinction experiments, both quinine and sinigrin were shown to be aversive. All together, the two experiments showed that both sinigrin and quinine can be aversive to *H. virescens*, with a more consistent effect of quinine relative to sinigrin. Furthermore, during the pre-exposure phase of experiment 3, 24.6% of the insects showed PER to sinigrin stimulation, whereas only 3.5% showed PER to quinine stimulation, supporting the assumption of a stronger aversiveness to quinine. In previous feeding and proboscis extension experiments, sinigrin has been shown to be non-appetitive for *H. virescens* (Blaney and Simmonds, 1988; Jørgensen et al., 2006), but the behavioural effect of quinine has not previously been assayed in this moth. The increasing elicitation of PER to sinigrin during the pre-exposure phase could be due to a familiarity of the substance after several exposures to the moths. Since the substance is not toxic (the moths ingesting it survived), the moths might have learned that sinigrin is harmless in spite of the bitter taste. Insects have evolved a variety of physiological mechanisms for selectively adapting their aversive responses to harmless or toxic substances (Glendinning and Gonzalez, 1995). In contrast, bitter taste thresholds in mammals vary independently of toxicity thresholds, indicating that the bitter rejection response is just as likely to be elicited by a harmless bitter food as it is by a harmful one (Glendinning, 1994). In our experiment, another possibility is that the 2-day starvation period before the experiment, which is necessary for PER conditioning in moths, might have caused the insects to elicit PER to substances they would normally avoid.

In the acquisition phase following the pre-exposure phase (experiment 3), we found that previous presentation of linalool (paired with the dry toothpick) caused significantly reduced acquisition performance relative to the untreated group. The dry toothpick elicits a mechanosensory response in the receptor neurons, but presumably this has neither an aversive nor an appetitive influence on the moth. Therefore, it is possible that this group shows a typical latent inhibition phenomenon that has previously been shown in a number of animals, like honeybees (Abramson and Bitterman, 1986; Chandra et al., 2001). If this is a pure CS pre-exposure effect is not known because there was no control with mechanosensory stimulation alone. During the repeated presentations of CS in the absence of a punishment or a reward, it is believed that the CS is associated with the absence of reinforcement, which leads to a resistance towards re-learning the CS as a predictor for a reward (or punishment) in the subsequent acquisition phase. Other interpretations propose that the CS becomes less and less surprising in the experimental context, and therefore loses meaning throughout the pre-exposure phase (learned inattention, Lubow, 1997). Most importantly, when the CS was

associated with quinine in the pre-exposure phase in our study, the acquisition deficit was significantly increased. In this case, it is possible that the moths built aversive associations between linalool (CS) and quinine as an aversive reinforcer. Thus, at the end of the pre-exposure phase, linalool predicted the presence of a negative stimulus, which had a stronger obstructing effect on acquisition than just an absence of a reward or punishment, as is the case with the mechanosensory treatment.

Quinine has previously been found to have an aversive, but not a reinforcing effect in associative learning in *Drosophila* larvae (Gerber et al., 2004; Hendel et al., 2005). However, conditioned inhibition of the proboscis extension in adult *Drosophila* was observed when the proboscis extension was punished by applying quinine to the foreleg tarsi (DeJianne et al., 1985), supporting that quinine can act as a negative reinforcer. Other experiments on adult *Drosophila* have also shown that quinine supports aversive association with olfactory or other gustatory stimuli (Mery and Kawecki, 2002). In differential conditioning of bumblebees, quinine acted as a negative reinforcer, enabling the insects to discriminate between visual stimuli faster than if the CS was just associated with an absence of reward (Chittka et al., 2003; Dyer and Chittka, 2004). Although our experiments showed that quinine had an aversive effect in moths, a definite proof for a negative reinforcing effect of quinine is still lacking, since we have not controlled for possible non-associative effects of quinine. However, repeated presentations of quinine, sinigrin and the dry toothpick did not seem to reduce the appetitive motivation compared to the untreated control. Future experiments including a pre-exposure phase where moths receive unpaired presentations of CS and quinine will constitute a control for the formation of aversive CS-quinine associations.

In experiment 3, the group receiving sinigrin treatment showed the same tendency towards reduced acquisition and retention as the quinine group, although its performance was not significantly lower than that of the control group. Possibly, testing an even larger number of animals, or presenting a higher concentration of sinigrin could have yielded a significant difference. To confirm a possible aversive effect of the two tastants, we performed facilitated extinction experiments (Fig 5), showing that both quinine and sinigrin enhanced extinction, compared to the control. As before, we may explain the results in terms of the formation of aversive associations. Thus, the moths would learn two associations after one another; during acquisition, they would form CS-sucrose associations acting positively on PER, and during the second phase, they would form CS-quinine or CS-sinigrin associations, causing a resistance to elicit PER. Responses would thus reflect a balance between the two types of associations, the aversive association progressively overbalancing the appetitive association.

Additionally, a second type of explanation could apply in the facilitated extinction experiment. Increased extinction with the bitter substances could be a form of operant learning, because the action of PER was punished by providing the bitter substance to the antennae and the proboscis. To test for such effects, adequate controls can be applied, like the use of omission and yoked groups, in which the bitter reinforcement of the moths would be uncoupled from the PER.

In both the pre-exposure and the facilitated extinction experiments, it was shown that quinine, and to a lesser extent sinigrin, detected by GRNs on the antenna, had aversive effects on the moth behaviour. Although it was not the aim of the present work to study aversive learning in moths, it is possible that the effect found of both impaired acquisition (experiment 3) and facilitated extinction (experiment 4), is caused by the formation of CS-bitter tastant associations. Choice tests could perhaps reveal such associations. For example, in a PER situation, one group of moths could be exposed to an odour combined with quinine or sinigrin, whereas another control group could be exposed to an odour of similar salience combined with no stimulus. If the treated moths in a subsequent choice test actively choose the odour combined with no stimuli, then a formation of CS-bitter tastant association could be proven. Another way of testing this would be to let the same moth receive one odour with quinine or sinigrin and another odour with no other stimulus in a PER situation, and subsequently let the moth choose between odours.

If quinine and sinigrin were negative reinforcers, we would expect that the reinforcement signals triggered by quinine and sinigrin would converge with the olfactory pathway to form associations in the moth, possibly involving a modulatory neuron with opposite effect to the VUMmx1 in honeybees. In honeybees (Vergoz et al., 2007) and in *Drosophila* (Schwaerzel et al., 2003), dopamine has been found to be the neurotransmitter involved in aversive olfactory learning with electric shock as punishment. In crickets (Unoki et al., 2005; Unoki et al., 2006), dopamine was involved in odour- and colour-salt punishment associations. Moreover, in *Drosophila* larvae, activation of dopaminergic neurons in association with an odour stimulus was sufficient to create an aversive olfactory memory (Schroll et al., 2006). All these data point towards a prominent role of dopaminergic modulatory neurons in odour-punishment associations, and in the formation of aversive olfactory memories. The confirmation of the existence of odour-bitter taste associations in moths and their dependency on such dopaminergic reinforcement systems will be the focus of future work.

Abbreviations:

CR: Conditioned response

CS: Conditioned stimulus

GRN: Gustatory receptor neuron

ITI: Inter-trial interval

PER: Proboscis extension response

UR: Unconditioned response

US: Unconditioned stimulus

VUM_{mx1}: Ventral unpaired median neuron of the maxillary neuromere 1

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Figure legends

Fig 1: The effect of US concentration on acquisition, retention and extinction of the conditioned PER, and the effect of time on retention and extinction in *H. virescens*. The proportion (%) of moths showing CR in each of the acquisition, retention, and extinction trials is shown. A: Average acquisition curves obtained in classical conditioning experiments with *racemic* linalool as CS and 2 M and 3 M sucrose as US. The letters NS indicate no significant between-group differences (Mann-Whitney test, $p < 0.05$). B: Retention in moths receiving 2 M or 3 M sucrose reward tested at different times after acquisition. Retention decreased significantly from 15 min to 48 h. $N > 31$ in all retention groups. Different letters indicate significant between-group differences (Fisher's exact tests, $p < 0.0127$). C: Acquisition and extinction curves for the five retention times and the two sucrose concentrations. The extinction curves were obtained by stimulating with CS alone. No significant between-group differences were found, indicated by the letters NS (2M: Kruskal-Wallis test, $p > 0.05$, 3 M: Kruskal-Wallis test, $p > 0.05$). D: Extinction curves for moths tested after 15 min, 2 h, 8 h, 24 h, or 48 h. Only moths showing CR at the first extinction test were included. Extinction was slower in the moths tested after 48 h. Different letters indicate significant between-group differences (Noether tests, $p < 0.0127$).

Fig 2: Typical responses obtained by tip recordings from gustatory receptor neurons in *s. chaetica* on the flagellum of the *H. virescens* antennae. Stimulation and recording starts simultaneously when the electrode is applied and ends when the electrode is removed,

meaning that only the stimulation period is shown. A: Responses to 1 mM quinine, 1 mM sinigrin, 100 mM sinigrin, and the electrolyte 10 mM KCl in the same *s. chaeticum*. B: Responses to 1 mM quinine in four different *s. chaetica*. C: Responses to 100 mM sinigrin in four other sensilla.

Fig 3: A: Average dose-response curves for quinine and sinigrin obtained during 5 s recordings from single *s. chaetica*. The average response to the electrolyte 0.01 M KCl is indicated as a reference. B: Average temporal response patterns for KCl, quinine and two concentrations of sinigrin, counted in 0.5 s bins in 75 *s. chaetica* during 5 s recordings. While 100 mM sinigrin elicited a high response frequency very shortly after application, responses to 1 mM quinine were bursts of activity distributed over the whole 5 s recordings. Different letters indicate significant between-group differences. The dotted squares show tests within the first and the third time bin, respectively. Letters behind the captions in B indicate differences between the average spiking activity during 5 s (Scheffé tests after ANOVA, $p < 0.01$).

Fig 4: Inhibitory learning effects of pre-exposure to linalool paired with a mechanosensory control, quinine or sinigrin on acquisition and retention. A: Responses to the mechanosensory stimulus, quinine and sinigrin during pre-exposure. The odorant linalool alone elicited no responses. Different letters indicate significant between-group differences (Mann-Whitney tests, $p < 0.05$). B: Effect of pre-exposure on acquisition in moths. The group of moths receiving quinine treatment showed lower acquisition than the control group, suggesting an aversive effect of quinine. Such an aversive effect appeared only as a tendency for sinigrin. The untreated group of moths was not pre-exposed. The control group showed reduced acquisition compared to the untreated group, corresponding to a latent inhibition effect. Different letters indicate significant between-group differences (Mann-Whitney tests, $p < 0.05$). C: The control group showed higher retention than the quinine treatment group, but not the sinigrin treatment group. The control group was not different from the untreated group in retention. Different letters indicate significant between-group differences (Fisher's exact tests, $p < 0.05$).

Fig 5: Acquisition, extinction and facilitated extinction of CRs in moths receiving different treatments during the extinction phase. A: Acquisition and extinction in moths receiving different extinction treatments. No significant between-group differences were found,

indicated by the letters NS (Mann-Whitney tests, $p > 0.05$). B: Extinction curves for moths that have learned the CS. Only moths showing CR at the first extinction test were included. Pairing of linalool with quinine or sinigrin induced a more rapidly decreasing number of responses than the control. Different letters indicate significant between-group differences (Mann-Whitney tests, $p < 0.05$).

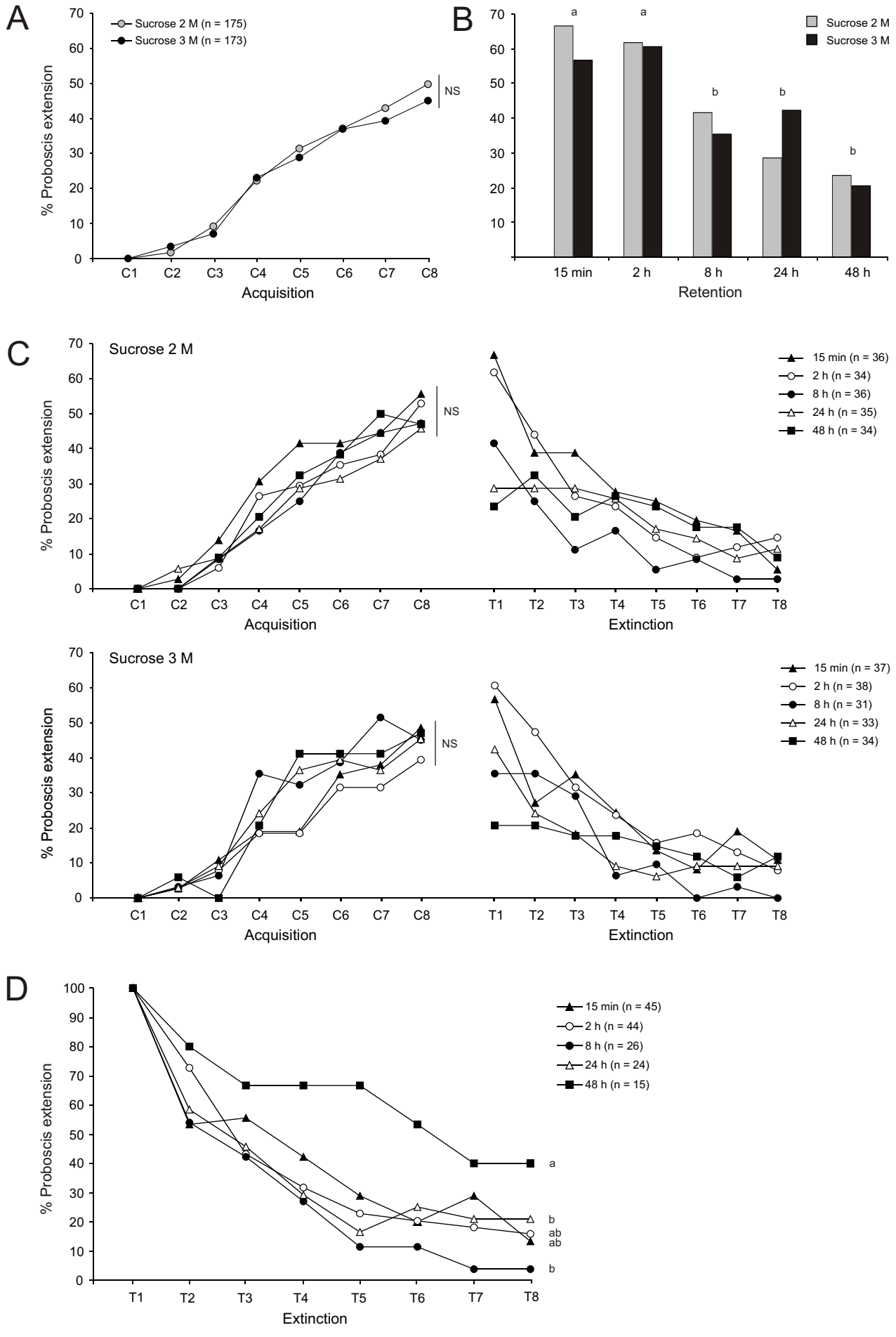
A
Sucrose 2 M (n = 175)
Sucrose 3 M (n = 173)
% Proboscis extension
NS
C1 C2 C3 C4 C5 C6 C7 C8
Acquisition
B
Sucrose 2 M
Sucrose 3 M
a a b b b
15 min 2 h 8 h 24 h 48 h
Retention
C
Sucrose 2 M
15 min (n = 36)
2 h (n = 34)
8 h (n = 36)
24 h (n = 35)
48 h (n = 34)
NS
C1 C2 C3 C4 C5 C6 C7 C8
Acquisition
T1 T2 T3 T4 T5 T6 T7 T8
Extinction
Sucrose 3 M
15 min (n = 37)
2 h (n = 38)
8 h (n = 31)
24 h (n = 33)
48 h (n = 34)
NS
D
15 min (n = 45)
2 h (n = 44)
8 h (n = 26)
24 h (n = 24)
48 h (n = 15)
a
b
ab
ab
b
T1 T2 T3 T4 T5 T6 T7 T8
Extinction

Figure 1

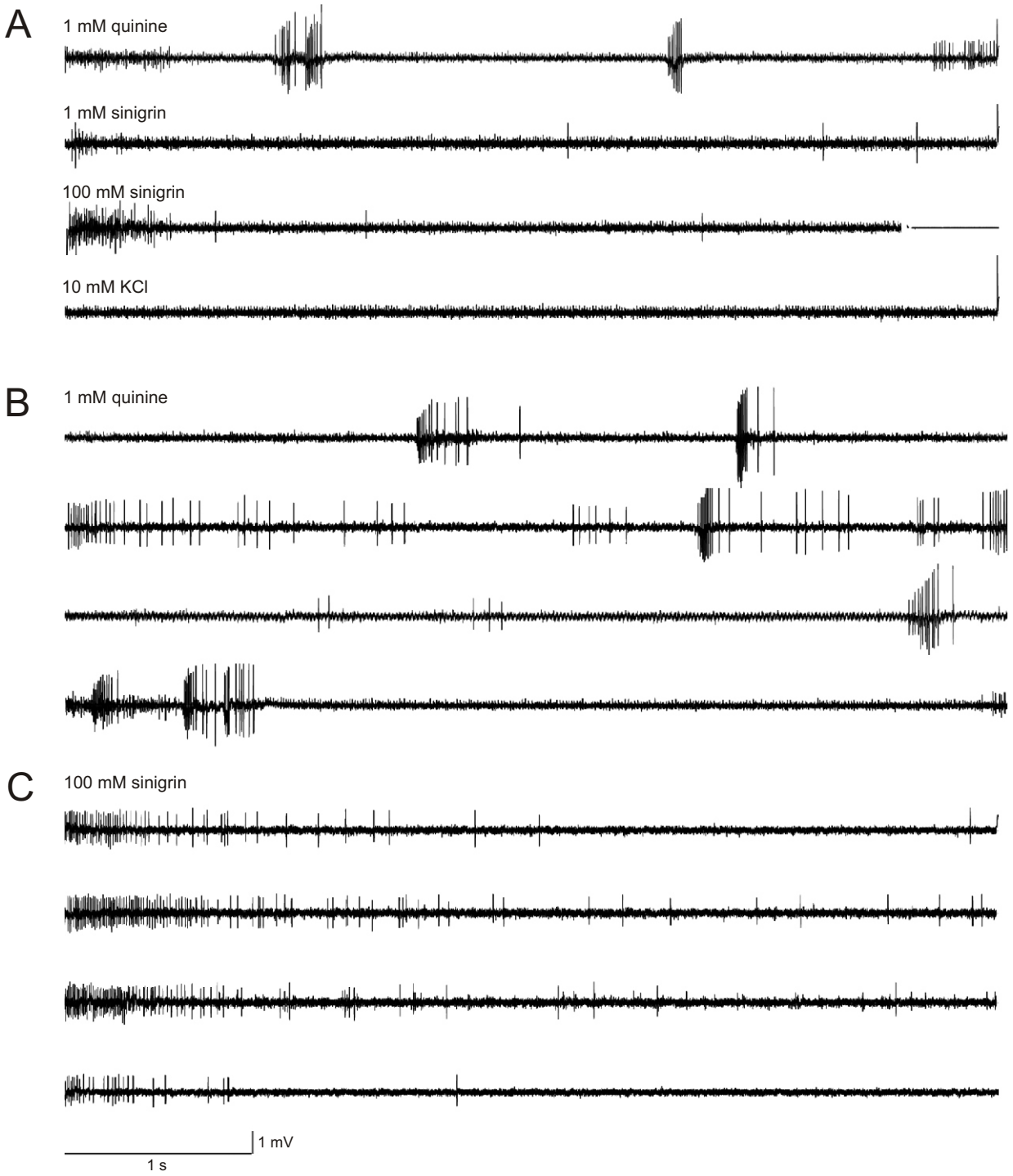
A
1 mM quinine
1 mM sinigrin
100 mM sinigrin
10 mM KCl
B
1 mM quinine
C
100 mM sinigrin
1 mV
1 s

Figure 2

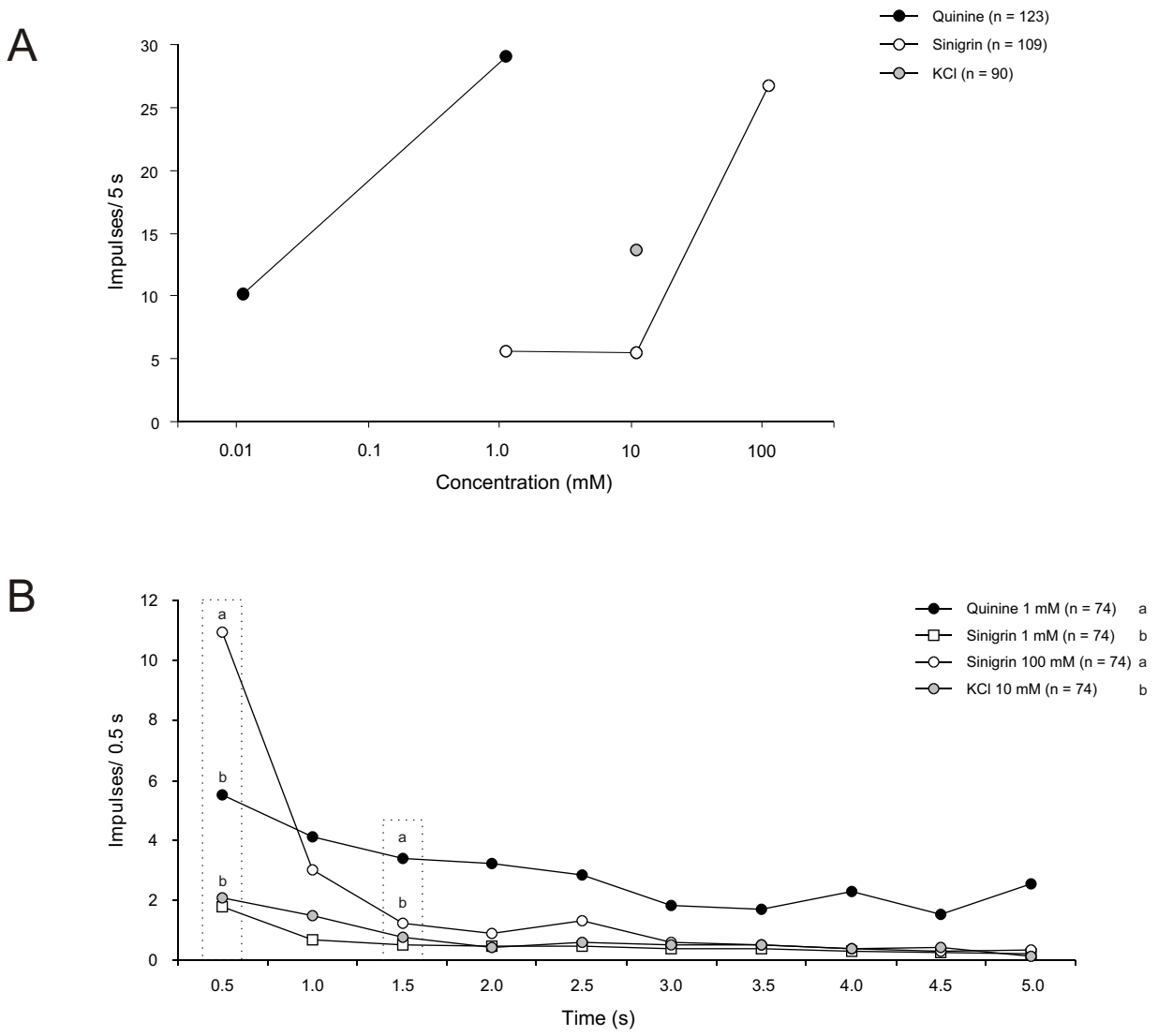
A
Quinine (n = 123)
Sinigrin (n = 109)
KCl (n = 90)
Impulses/ 5 s
0
5
10
15
20
25
30
0.01
0.1
1.0
10
100
Concentration (mM)
B
Quinine 1 mM (n = 74) a
Sinigrin 1 mM (n = 74) b
Sinigrin 100 mM (n = 74) a
KCl 10 mM (n = 74) b
Impulses/ 0.5 s
0
2
4
6
8
10
12
a
b
a
b
b
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Time (s)

Figure 3

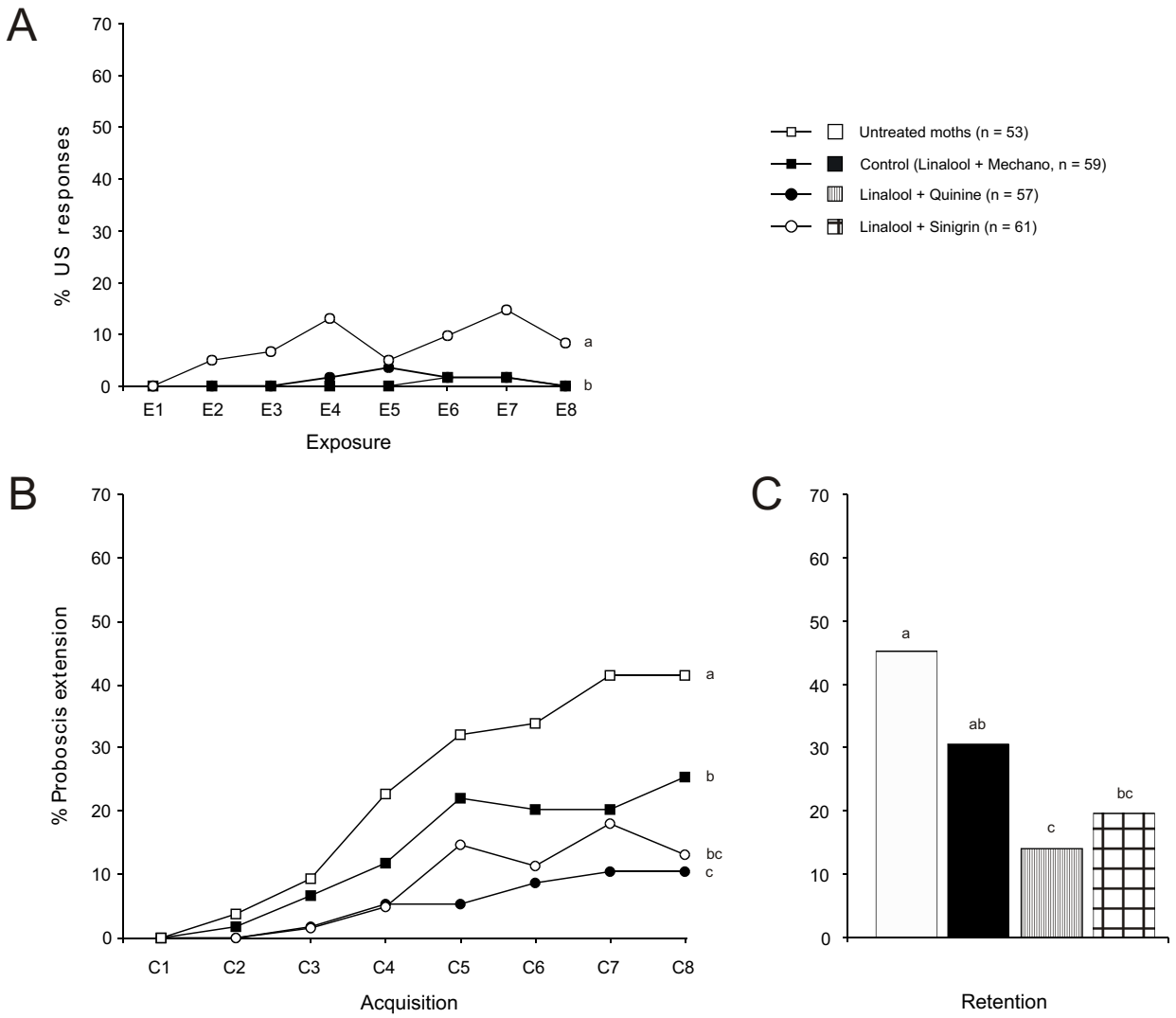
A
% US responses
70
60
50
40
30
20
10
0
E1
E2
E3
E4
E5
E6
E7
E8
Exposure
a
b
Untreated moths (n = 53)
Control (Linalool + Mechano, n = 59)
Linalool + Quinine (n = 57)
Linalool + Sinigrin (n = 61)
B
% Proboscis extension
C1
C2
C3
C4
C5
C6
C7
C8
Acquisition
a
b
bc
c
C
Retention
a
ab
c
bc

Figure 4

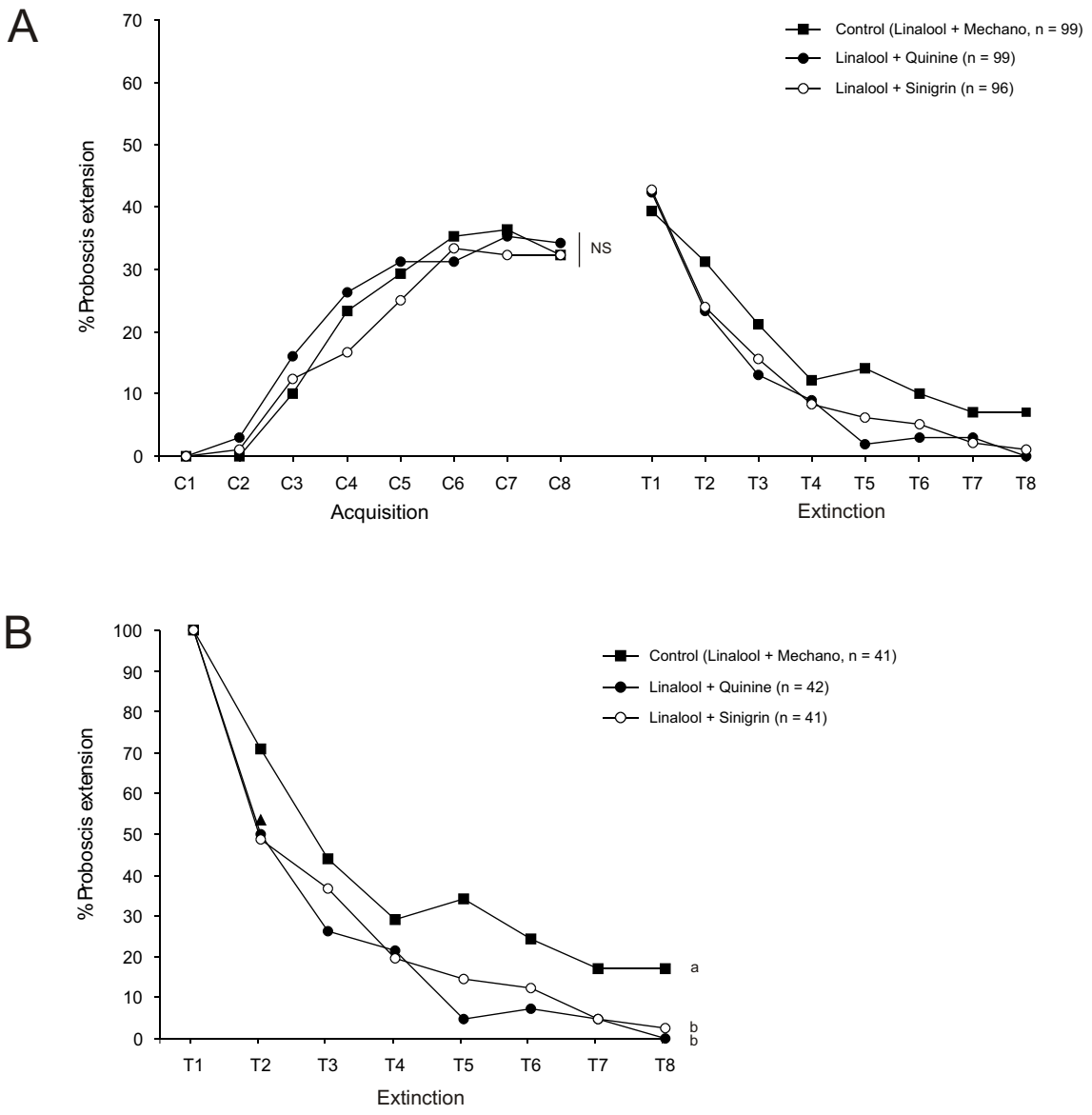
A
Control (Linalool + Mechano, n = 99)
Linalool + Quinine (n = 99)
Linalool + Sinigrin (n = 96)
% Proboscis extension
NS
C1 C2 C3 C4 C5 C6 C7 C8
Acquisition
T1 T2 T3 T4 T5 T6 T7 T8
Extinction
B
Control (Linalool + Mechano, n = 41)
Linalool + Quinine (n = 42)
Linalool + Sinigrin (n = 41)
% Proboscis extension
a
b
b
T1 T2 T3 T4 T5 T6 T7 T8
Extinction

Figure 5

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus.*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.
1987	Jarle Inge Holten	Dr. philos Bothany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988	Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988	Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988	Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988	Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
1989	John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989	Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
1989	Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990	Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990	Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
1990	Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990	Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
1991	Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway.
1991	Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient. Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.

1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
1991	Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992	Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses

1994	Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
1994	Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cockoo.
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995	Hanne Christensen	Dr. scient. Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
1995	Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
1995	Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995	Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden.
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae.
1996	Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
1996	Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996	Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.
1996	Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae.
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.

1997 Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997 Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997 Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997 Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*.
1997 Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos. Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998 Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998 Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998 Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach.
1998 Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999 Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999 Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999 Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.

1999	Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999	Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999	Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway

2000 Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001 Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001 Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001 Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001 Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002 Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002 Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002 Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002 Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002 Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002 Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002 Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002 Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002 Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002 Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003 Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003 Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003 Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003 Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003 Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003 Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003 Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003 David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003 Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective

2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations.
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations

2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Ander Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	phD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	phD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	phD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	phD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	phD Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	phD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	phD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation