

Synthesizing Neurophysiology, Genetics, Behaviour and Learning to Produce Whole-Insect Programmable Sensors to Detect Volatile Chemicals

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Abstract

Insects have extremely sensitive systems of olfaction. These systems have been explored as potential sensors for odourants associated with forensics, medicine, security, and agriculture application. Most sensors based on insect olfaction utilize associative learning to “program” the insects to exhibit some form of behavioural response to a target odourant. To move to the next stage of development with whole-insect programmable sensors, an examination of how odourants are captured, processed and used to create behaviour is necessary. This review article examines how the neurophysiological, molecular, genetic and behavioural system of olfaction works and how an understanding of these systems should lead the way to future developments in whole-insect programmable sensors.

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Abbreviations: ORNs, olfactory receptor neurons; AL, antenna lobes; OB, olfactory bulb; PN, projection neuron; US, unconditioned stimulus; CS, conditioned stimulus; CR, conditioned response; PER, proboscis extension response; EAG, electroantennogram; GL, glomeruli; LIDAR, light detection and ranging; GPCR, G protein-coupled receptor; ODE, ordinary differential equation.

Introduction

Most animals have evolved highly sensitive olfactory systems which respond to odours in their environments. Insects in particular must navigate their environment using visual, olfactory, and tactile signals received and processed by their sensory systems to control behavioural responses to food, predators, mates or hosts necessary for individual and collective survival. Insects have very robust and extremely sensitive olfactory systems with extraordinarily high discriminating abilities to identify specific odours with only a small number of molecules (Angioy *et al.*, 2003). There are a number of known examples in which olfaction is known to play a significant role in the behaviour of insects. Some of the more interesting are: The malarial-vector mosquito *Anopheles gambiae* detects their human host by body odours and CO₂ (Takken, 1996); the males of the sphinx moth *Manduca sexta* use pheromones released by female to find mates (Hildebrand, 1995); the honeybee *Apis mellifera* learns different odours to form navigational memories and use them for foraging behaviour (Galizia and Menzel, 2000; Reinhard *et al.*, 2004); and parasitic wasps, such as *Microplitis croceipes*, detect odours produced by plants in response to host feeding (Lewis and Takasu, 1990).

Learning can be defined as a process by which a change in behaviour is exhibited as a result of experience (Thorpe, 1963). The extremely sensitive nature of the insect olfaction system is enhanced by the ability to learn to associate external stimuli with resources, such as food, hosts, and mates. Associative learning in insects has been studied using classical and operant conditioning. The sophisticated ability of honeybees to learn through the notable work of von Frisch (1915, 1950) and Menzel (1985) and more recently the studies of learning in parasitoid wasps and fly species (reviewed in Vet *et al.*, 1995) have been extensively studied. In addition, insects can be trained to associate simultaneously two different odours with food and host rewards (Lewis and Takasu, 1990) or successively to two different odours with the same reward (e.g., Takasu and Lewis, 1996). This training is particularly simple for some parasitic wasps; they can be trained within 5 minutes (Lewis and Takasu, 1990; Takasu and Lewis, 1996). There are three primary factors in associative learning that affect how well insects are conditioned to an single odour source. These are the number of trials the insect is provided an odour [conditioned stimulus (CS)] immediately followed by feeding on sugar water [reward or unconditioned stimulus (US)], the length of time the insect is given food while exposed to the odour and the time interval between each trial. Another significant factor in the level of conditioning received is the physiological state of the insect; Starved parasitic wasps have been conditioned using food as the unconditioned stimulus, while satiated wasps were shown to be more easily conditioned using host frass as the US (Lewis and Takasu, 1990). This and other studies on associative learning in insects have led to the generally accepted conclusion that associative learning is a ubiquitous trait in insects (Dukas, 2008).

There have been a few studies, including those by the authors, which have demonstrated the ability to condition wasps, bees, and moths to odours of human interest and to detect these odours through measurable behavioural responses. The conditioned response (CR), such as the proboscis extension response (PER) and area-restricted searching are used as active feedback indicators of the presence of the odours of interest. Examples include conditioning parasitic wasps, moths and honey bees to

chemicals associated with explosives, food toxins and plant odours. In these studies, insects are contained and observed using electronic interfaces that measure behavioural responses, or released and allowed to search and find an odour source.

There are many characteristics of insect olfaction that makes them useful as chemical detectors, some of which are: 1) the ability to detect low levels of chemical odours (Rains *et al.*, 2004), 2) the ability to detect and respond to odours in a background of non-target odours (Rains *et al.*, 2006), 3) a short generation time which allows many life cycles to be produced in a short period of time, 4) the ease of rearing large numbers, 5) the great diversity of insects which allows us to draw upon different species for use in specific habitats or environments (e.g. flying, ground-dwelling or aquatic, nocturnal or diurnal, piercing-sucking or chewing mouthparts, large or small-sized), and 6) the ability to be conditioned to odours in a matter of minutes.

During the last three decades discoveries in anatomy, neurophysiology and molecular biology that underlie insect olfactory systems have allowed us to uncover, for example, the functionalities of the various components of the olfaction system and some of the mechanisms underlying olfactory memory formation. These discoveries have, however, led to new questions such as: What, where, and how is odourant information processed and stored? How reliable is the information stored and for how long a period can the information be stored? How are memories synthesized with acquired information, either through learning or experience? How does odour discrimination affect behavioural control in the brain? Where do cellular memory traces occur within the olfactory nervous system in response to learning? The answer to these questions and others are difficult challenges. From the time a molecule is captured by odourant binding proteins in the antennal sensillum, to the body movements elicited by motor neurons as a behavioural response, we seek a better understanding of how sensory signals cause behaviour and how behaviour controls sensory processing. Through a clearer understanding of how the behavioural, physiological, genetic and molecular levels interact, we believe that whole-insect programmable sensors can be developed for chemical detection, and serve as models for development of better electronic chemical sensing systems as well.

Current whole-insect programmable sensors

Whole-insect programmable sensors were initially investigated under military funding to detect landmines, explosives and toxins. However, additional application areas are numerous and include, but are not limited to such things as detection of leaks, food toxins, plant and animal disease, drugs, cadavers, gravesites, accelerants used for arson, termite infestations, and minerals.

Insect chemical detectors have been developed and reported using honey bees, hawk moths and parasitic wasps. Honey bees are easily reared and studies of their learning and sensing abilities are extensive, providing much background information. The hawk moth is of interest in agriculture as a pest in the larval stage and its large size has made it a useful model for examining the olfactory system. Parasitic wasps have been studied as beneficial insects in agriculture and their tri-trophic interaction with plants and hosts has revealed an intricate foraging strategy for finding food and hosts.

Honey bees learning ability, and memory of learned stimuli have been examined to determine their ability to forage and navigate landscapes using visual and olfac-

tory cues. These studies have proved useful in determining how they may be used as chemical sensors. Recent studies have examined the discriminatory abilities of honey bees to changing concentrations of odourants the bees were trained to detect along with chemicals of similar molecular structures (Wright and Smith, 2004; Wright *et al.*, 2005). Interestingly, honey bees classically conditioned to a low concentration of a target odourant are able to distinguish that odour from molecularly similar odours; but, recognize these odours as the same when conditioning at higher concentrations. It was concluded that odour intensity may be a salient feature of the odourant at low concentration, but not at higher concentrations. Honey bees also appeared to generalize the identity of an odourant mixture using one odour within that mixture, as long as it remained constant during conditioning. Test trials were conducted with all odours in the mixture but one varied during conditioning. Subsequent tests with individual odours found the strongest PER to the odour that was held constant. A related study also found that at lower concentrations, the time the bee is allowed to sample the odour while feeding on sugar water increases their ability to distinguish that odourant (Wright *et al.*, 2009). Other important studies have examined the effects of latency, overshadowing and blocking of conditioned odours within odour mixtures (Hosler and Smith, 2000, Linster and Smith, 1997). These and other studies provide strong background for how to implement conditioning and testing mechanisms in whole-insect programmable sensors.

In most studies of the response to conditioning, honey bees are held in a harness and the PER measured as odours are passed over the antennae. This method is also utilized in a commercial device developed by Inscintinel Ltd. Other methods have examined the use of free-flying bees and light detection and ranging (LIDAR) to track bee location (Hoffman *et al.*, 2007).

M. sexta, has also been studied extensively and has provided some very useful insights into the physiology and biological processes that govern insect olfaction (reviewed later). A device using 10 hawkmoths was developed to electrically monitor the feeding muscles to determine when cyclohexanone was detected (King *et al.*, 2004). Although this device was too large as built for easy transportation, it would be trivial to miniaturize to proportions that make it portable. Also an electroantennogram (EAG) quadroprobe device utilizing the antennae and whole organism of two moth species, *Helicoverpa zea* and *Trichoplusia ni*, has been developed and tested to detect odour plumes (Myrick *et al.*, 2009, Park *et al.*, 2002). Antennae are either excised or used directly on the insect and EAG response recorded and analyzed, leaving the interpretation of the raw signals to analysis software. Using whole insects, the probe response was conserved for up to 24 hours and was able to classify individual odourant plumes in less than 1 second.

M. croceipes has been studied for its ability to forage effectively for hosts and nectar sources. Several studies have examined the wasp's ability to learn, retain, discriminate, and respond to odourants, both within and outside their natural habitat (Takasu and Lewis, 1996). *M. croceipes* has also been tested against the electronic nose, Cyranose 320 (Smiths Detection, Inc.) and was found to be almost 100 times more sensitive to the chemicals, myrcene and 3-octanone (Rains *et al.*, 2004). In the case of this parasitic wasp, a positive response to the target volatile chemical is measured by a movement of the wasp body and antennae around the location of the odour source when the unconditioned stimulus is food. Parasitic wasps were also

demonstrated to respond with context-dependent behavioural movements depending on the resource the odourant was linked to (Olson *et al.*, 2003). *M. croceipes* were conditioned to an odourant while feeding on sugar water. After a minimum of 15 minutes, the same wasps were conditioned to a different odourant while stinging their host, *H. zea*. Consequently, when each wasp was presented with one of the conditioning odourants, the wasps responded with either foraging behaviour (food) or a stinging behaviour (host). Further studies have revealed that *M. croceipes* can also discriminate molecularly similar odourants (Meiners *et al.*, 2002). Further studies are needed to understand the affect of odour mixtures and when an odour becomes unrecognizable to the olfactory sensory system.

To utilize the parasitic wasp as a sensor, a device called the Wasp Hound, was developed and has been used to detect the odours associated with Aflatoxin in foods (Rains *et al.*, 2006) and animal carcasses (Tomberlin *et al.*, 2008). Wasps are placed in a small cartridge and sample air passed through. A web camera records images of wasp behaviour and the Wasp Hound connected to a software program called Visual Cortex that analyzes the wasp response and indicates the response as a real-time graph (Utley *et al.*, 2007).

Future developments

The above described examples of whole-insect programmable sensors, with the exception of the EAG quadroprobe, use crudely adapted methods of direct behavioural response to determine when the insect has detected a target odourant. For example, the Wasp Hound measures the crowding of wasps response to the presence of the target odourant around the inlet where the sample air is pumped into the device (Rains *et al.*, 2006). However, the entire repertoire of behaviours also encompasses the initial recognition of the odour, its quality, and possibly its quantity (concentration) as well. As discussed with honey bees, odourant concentration is a salient property, at least at low concentrations. However, the PER response may also be dependent on the perception of whether the odour source is near or far. A PER response by honey bees would necessarily be perceived as a direct contact with the odour source (close proximity to odour source). Consequently, other behavioural responses may be more evident when the odour is perceived to be a distance away. The basic hypothesis is that odour characteristics (quality and quantity), along with what has already been learned and stored in memory control the signals to motor neurons that control movement and behaviour. If behaviours could be observed and analyzed to determine what sensory characteristics caused that behaviour, insect sensors could be used to measure concentrations and track odours to the source (Rains *et al.*, 2008).

To adapt systems that extract this information from a whole-insect programmable sensor, more targeted research is necessary that examines the processes that direct learned behaviour at the molecular and genetic level. Neuronal signals to the mushroom bodies and lateral horn (*Figure 1*) that are derived from learning new experiences, combined with memory of past experiences in the brain centre, results in signals to motor neurons that result in complex behaviours. Determining the how and why a specific behaviour occurs at the molecular level should lead to methods to better utilize the insect olfaction system as a chemical detector. To that end, we will now

review the olfactory system of insects and computational models that examine the process of olfaction. As part of that review, we will introduce potential opportunities and obstacles for chemical detection system development.

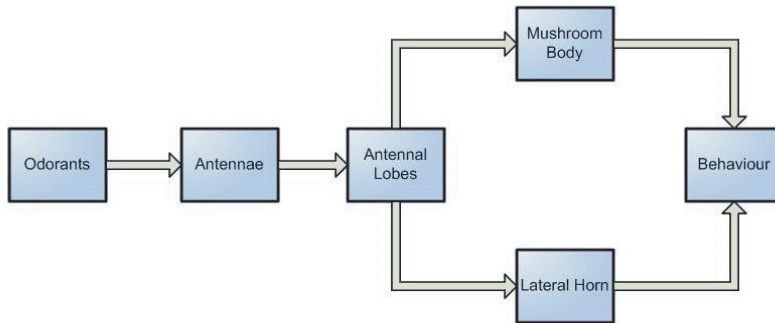


Figure 1. Organization of the insect olfactory system. The first level of odour processing in insects occurs at the antennae. Olfactory receptor neurons (ORNs) inside the insect antennae are compartmentalized into sensory hairs called sensilla (each sensillum contains the dendrites of up to four ORNs). The odourants in the air enter the sensillum through pores in the cuticle of the antennae. Then the ORNs in the sensillum generate odour specific electrical signals (called spikes) in response to the odour. The axons of the ORNs join to the antennal nerve, and project to the antennal lobe. The future processing of the olfactory information occurs in the antennal lobe before it is sent to protocerebrum. The antennal lobe contains globular shaped structures called glomeruli. They are the projection fields of the olfactory receptor neurons onto the second order neurons, which are called projection neurons (PNs). The glomeruli also contain the processes of local interneurons that branch to multiple glomeruli, and transfer the olfactory information between glomeruli. Individual ORNs send axons to only one or a few glomeruli, and individual PNs typically innervate only one single glomerulus. The axons of PNs project to the mushroom body (MB) and lateral horn of the brain. The mushroom bodies are located in the protocerebrum and are the centres of higher order processing in insects. They are a paired structure consisting of thousands of small intrinsic nerve cells (Kenyon cells). These Kenyon cells have their projections within mushroom body structure. They receive sensory information via the dendritic calyx, and send axonal projections to the anterior brain where they bifurcate to form the medial and vertical lobes. The mushroom bodies are also involved in olfactory memory formation. The lateral horn is the PN axon terminal field and is involved in odour recognition.

Features of insect olfactory system

In general, insects use their antennae for olfaction and olfactory receptor neurons (ORNs) in the antennae generate odour-specific electrical signals called spikes (Hallem and Carlson, 2004). These spikes can be recorded using an EAG and have been used to understand how odourants initiate signals in the olfactory system. A signal pathway model given in *Figure 1* has four distinct components: antennae, antennal lobes, mushroom bodies and lateral horn. The antennae have the sensory neurons in the sensilla hairs and their axons (*Figure 2a*) terminating in the antennal lobes (AL) where they synapse with the neurons in the spherical units (*Figure 2b*) called glomeruli (GL). These AL's have two kinds of neurons, excitatory projection neurons (PN) and inhibitory local neurons.

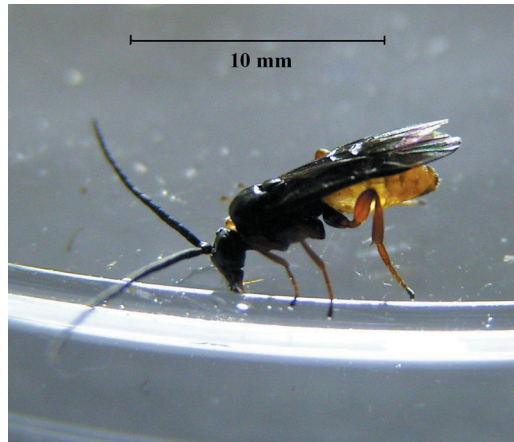


Figure 2a. A parasitic wasp, *Microplitis croceipes*. Olfactory receptor neurons (ORNs) are compartmentalized into sensory hairs called sensilla on the surface of the antennae.

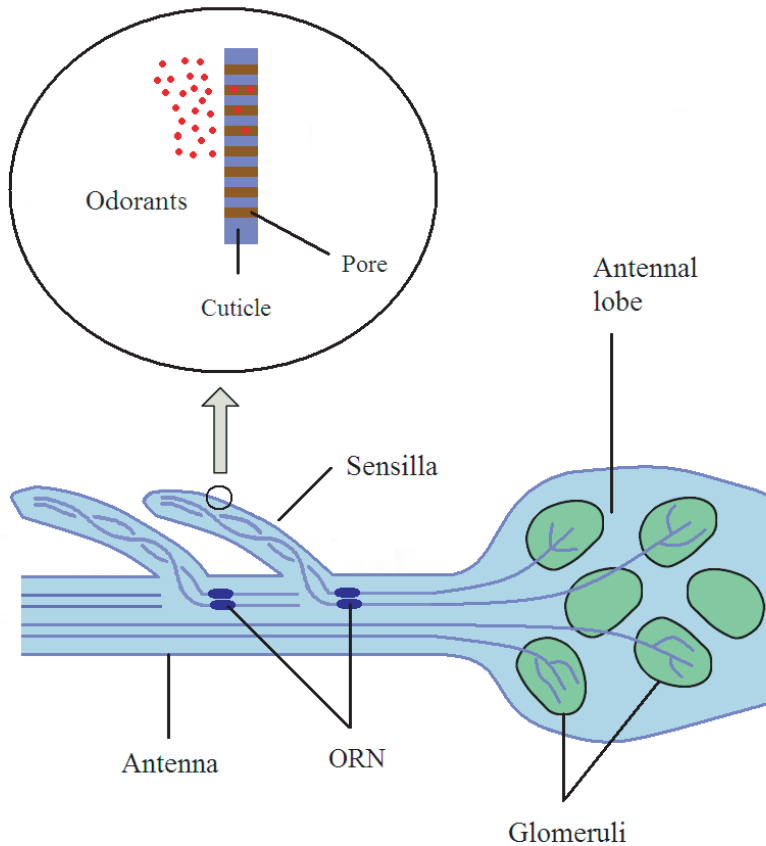


Figure 2b. Insect olfactory sensilla. The ORNs are compartmentalised into sensory hairs to detect **odorants** which are called sensilla. Each sensillum contains the dendrites of up to four ORNs. The sensillum has three major morphological types: basiconic, coeloconic and trichoid. **Odorants** in the air enter the sensillum through pores in the cuticle. ORNs project their axons to glomeruli in the antennal lobe.

The projection neurons send their axon terminals to the mushroom body (MB) and lateral horn (LH) both of which are part of the protocerebrum of the insects (*Figures 3 and 4*). The inhibitory local neurons have no axons. Recordings from projection neurons show in some insects strong specialization and discrimination for the odours presented (e.g., the projection neurons of the macroglomeruli, a specialized complex of glomeruli responsible for pheromone detection). How information passed to the mushroom bodies is stored and processed to in-turn send signals via motor neurons to muscles that control complex behaviour is not exactly known though some preliminary studies shed some light on the subject. How this occurs will unlock a key component in understanding the basis for behavioural responses to resource needs, such as food and mates. This in turn is important to understanding and developing sensors that can, from behavioural cues, know what sensory inputs are causing them. Model organisms such as the fruit fly, *Drosophila melanogaster*, help develop general conceptual models of olfactory systems and their operational principles; but each type of insect has its own systemic specificities. For example, *Drosophila* antennae have approximately 1200 ORNs, and the maxillary palps 120 (Shanbhag *et al.*, 1999, 2000; Stocker, 1996).

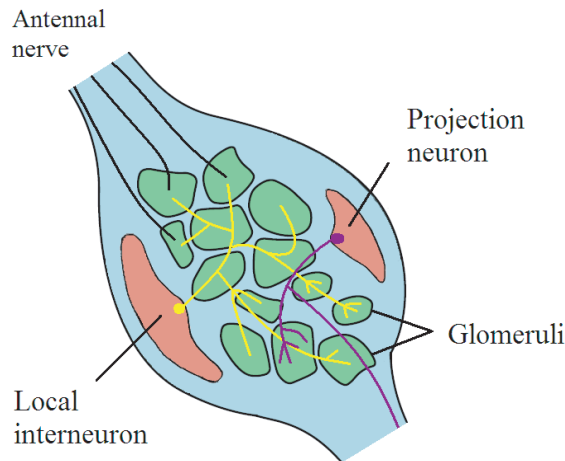


Figure 3. The insect antennal lobe. ORNs send axons from the insect antenna to the antennal lobe via the antennal nerve. ORNs synapse onto second order neurons called projection neurons (PNs) in the AL. The AL can be subdivided into spherical units called glomeruli. Individual ORNs send axons to only one or a few glomeruli, and individual PNs typically innervate only a single glomerulus. The glomeruli also contain the processes of local interneurons that branch in multiple glomeruli, and transfer information between glomeruli. The projection neurons project to higher brain centres such as the mushroom body and lateral horn of the protocerebrum. The local neurons, which are primarily inhibitory, have their neurites restricted to the antennal lobe.

The sensilla hairs in the antenna are categorized into basiconic, coeloconic and trichoid morphological types, the dendrites of up to four ORNs occupy each sensilla. The AL can be subdivided into around 43 GLs, and axons from each ORN can connect to only one or a few glomeruli, and an individual PN typically innervate only a

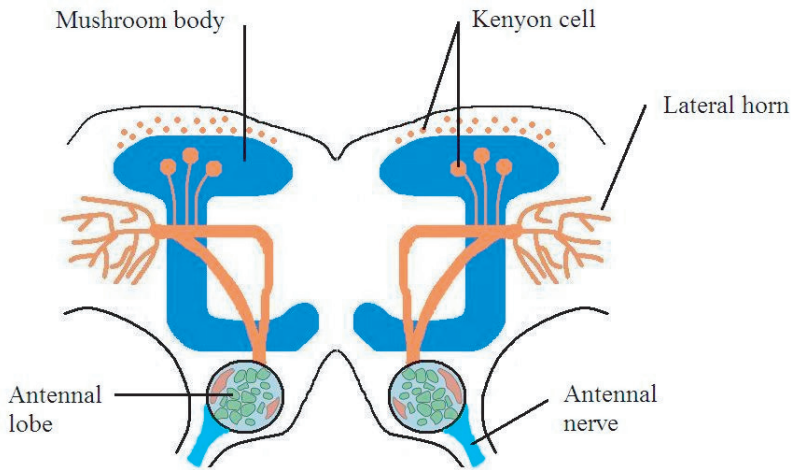


Figure 4. Horizontal section through the brain of the insect showing the main elements of the olfactory pathway. The antennal lobes and their glomeruli are in green, the mushroom bodies are in blue, and the Lateral horns are in orange. Odours are detected by olfactory receptor neurons (ORNs) in the antennae. The ORNs project axons along the antennal nerve to the antennal lobe glomeruli, where they are sorted according to chemo-sensitivity. From there the information is relayed by projection neurons (PNs) to the mushroom body and to the lateral horn. Some PNs bypass the mushroom body calyx and project only to the lateral horn.

single glomerulus (Jefferis *et al.*, 2001; Marin *et al.*, 2002; Wong *et al.*, 2002). The glomeruli also contain the processes of local interneurons that branch to multiple glomeruli (Stocker, 1996; Stocker *et al.*, 1990), providing means for information transfer between glomeruli. The axons of PNs project to the mushroom body (MB) and lateral horn of the brain.

Larvae of *Drosophila* also exhibit a robust olfactory response (Ayyub *et al.*, 1990; Cobb *et al.*, 1992; Monte *et al.*, 1989), which is mediated through the dorsal organ (Heimbeck *et al.*, 1999; Oppliger *et al.*, 2000). Each of the paired dorsal organs contains 21 neurons that project to the antennal lobe of the larval brain (Python and Stocker, 2002).

The ORNs of the antenna and maxillary palp generate action potentials in response to odour stimulation. The odour responses of many of these ORNs have been characterized through extracellular single-unit recordings from individual olfactory sensilla (Clyne *et al.*, 1997; de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001; Stensmyr *et al.*, 2003). These recordings have revealed that different odourants elicit responses from different subsets of ORNs, and also that ORNs exhibit a remarkable diversity of response properties: responses can be either excitatory or inhibitory and can vary in both intensity and temporal dynamics, depending on the odorant and the ORN (de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001). Similar ORN response properties have been described in other insects (Heinbockel and Kaissling, 1996; Kaissling *et al.*, 1989; Nikonov and Leal, 2002; Shields and Hildebrand, 2001). Extensive recordings from the antennae and maxillary palps have revealed that ORNs can be categorized into a limited number of functional classes based on their responses to a defined set of chemical odourants. The maxillary palp contains six functional classes of ORNs,

which are found in stereotyped pairs within three classes of sensilla (de Bruyne *et al.*, 1999). The antennal basiconic ORNs falls into 18 functional classes that are also found in stereotyped combinations within eight classes of sensilla (de Bruyne *et al.*, 2001; Elmore *et al.*, 2003); the coeloconic and trichoid sensilla on the antenna also contain multiple classes of ORNs (Clyne *et al.*, 1997) but a thorough characterization is not yet available.

The projection neurons project to higher brain centres such as the mushroom body and lateral horn of the protocerebrum. The local neurons, which are primarily inhibitory, have their neurites restricted to the antennal lobe. In *Drosophila*, each olfactory receptor neuron generally expresses a single olfactory receptor gene, and the neurons expressing a given gene all transmit information to one or two spatially invariant glomeruli in the antennal lobe. Moreover, each projection neuron generally receives information from a single glomerulus. The interaction between the olfactory receptor neurons, local neurons and projection neurons reformats the information input from the receptor neurons into a spatio-temporal code before it is sent to higher brain centres.

The mushroom bodies or corpora pedunculata are a pair of structures in the brain of insects and other arthropods. They are usually described as neuropils, i.e. as dense networks of neurons and glia. They get their name from their roughly hemispherical calyx, a protuberance that is joined to the rest of the brain by a central nerve tract or peduncle. Mushroom bodies are known to be involved in learning and memory, particularly for smell. They are largest in the Hymenoptera (bees, wasps, etc.) which are known to have particularly elaborate olfactory control over behaviour. The mushroom bodies have been compared to the cerebral cortex of mammals and are currently the subject of intense research. Because they are small compared to the brain structures of vertebrates, and yet many arthropods are capable of quite complex learning, it is hoped that investigations of the mushroom bodies will allow a clearer view of the neurophysiology of animal perception and cognition. The most recent research is also beginning to use new tools to reveal the genetic control of processes within the mushroom bodies (e.g. Olsen and Wilson, 2008). Also, through classical conditioning, the mushroom bodies incorporate the coded odourant signals from the antennal lobes and **make and association** between that code and the resource with which it was associated. Future studies that link the behavioural response with the neural signals and genetic expression in the mushroom bodies before and after associative learning could be used to better understand and condition insects and potentially other animals to odours. It would also lead to a basic understanding of how animals navigate through their environment.

Most of our current knowledge of the mushroom bodies comes from studies of a few species of insects, especially the cockroach *Periplaneta americana*, the honey bee *Apis mellifera*, the locust, *Schistocerca americana*, and the fruit fly, *Drosophila melanogaster*. Studies of fruit fly mushroom bodies have been particularly important for understanding the genetic basis of their functioning, since the genetics of this species are known in exceptional detail. In the insect brain, the peduncles of the mushroom bodies extend through the midbrain. They are mainly composed of the long, densely packed nerve fibres of the Kenyon cells, the intrinsic neurons of the mushroom bodies. These cells have been found in the mushroom bodies of all species

that have been investigated, though their number varies; for example fruit flies have around 2,500 and cockroaches have about 200,000.

Insects and other invertebrates do not have their olfaction system directly connected to respiration. As such, odours are not “sniffed”, but are brought into contact with ORN by antennal sensilla and behavioural movements. Insects in particular have a wide array of morphological features that make up the antennae. It is still unclear as to what evolutionary adaptations these features represent (Hansson *et al.*, 1991). However, it would be prudent to recognize the structural design and use of these organs as potential designs for improved detection of odourants using man-made sensing materials. Currently, electronic nose technology has focused on mimicking the combinatorial strategy that is well accepted as the mammalian and insect olfaction method of identifying odours from patterns of responses to different sensors (normally 16 or 32 sensors). However, little effort has examined the method in which odourant molecules are captured or presented to the sensors.

Biology of learning and memory

One of the important aspects of olfactory systems must be the ability to learn different odours and form memories of specific odours to mediate behavioural responses. Understanding the processes of learning and memory of odours could help improve methods of conditioning and measuring behavioural responses of whole-insect programmable sensors. To understand the physiological changes that occur in cellular and neuronal pathways, electrophysiological recordings have been used in mammalian model systems (Liu and Davis, 2006). Such studies are difficult to perform in insects because of their small size; therefore, functional optical imaging techniques have been employed to study physiological differences within the insect olfactory system. Synthetic chemical reporters and fluorescent proteins are used to report the activity of neurons in the system when an odour is introduced to an insect before or after olfactory learning has occurred (Faber *et al.*, 1999). In that work Faber and colleagues studied the honey bee antennal lobe using the calcium sensitive dye, calcium-green-2AM by observing the activity in response to odour stimuli presented before and after associative conditioning with sucrose as a rewarding stimulus. Specific areas of the antennal lobe exhibited calcium signals in response to odour, which increased for more than 30 min after conditioning. Learning changed the activation pattern in the antennal lobe. They discovered that the responses in specific sets of glomeruli were odour-specific, but conserved for each odour across honey bees. Protein-based reporters of neuronal activity were employed in recent studies, which have confirmed that memory traces form in the antennal lobes (Liu and Davis, 2006). However, these memory traces existed only for about five minutes after training corroborating the data from locusts (Bazhenov *et al.*, 2005) and moths (Daly *et al.*, 2004) which supports the hypothesis that the memory formation in antennal lobes are short-term.

The above mentioned studies and other recent studies show that olfactory memory is distributed among diverse types of neurons within the olfactory system, which are classified into first-, second- and third- order neurons (Davis, 2004). Perceptual olfactory learning happens in first-odour neurons which may be mediated by changes in the odourant receptive fields of second and/or third order neurons, and in the coherency

of activity among ensembles of second odour neurons (Davis, 2004). The coherent population activity of these neurons increases during operant olfactory conditioning. The odour responsiveness and synaptic activity of second and perhaps third order neurons increase during operant and classical conditioning (Davis, 2004).

Associative and non-associative processes influence odour-driven responses in the insect antennal lobe (AL). Daly *et al.* (2004) studied the changes in AL network activity during learning employing an *in vivo* protocol in *M. sexta* for continuous monitoring of neural ensembles and feeding behaviour over the course of olfactory conditioning. Daly *et al.* (2004) showed that the neural units in the AL responded to Pavlovian conditioning when odour followed food and the response persisted after conditioning. A net loss of neural units responding to odour occurred when odour did not predict food. The experiments showed that odour-specific neural recruitment was positively correlated with changes in the insect's behavioural response to odour. In addition, odour representations in the AL were dynamic and related to olfactory memory formation; learning continually restructures neural network responses spatially in the AL in an odour-specific manner (Daly *et al.*, 2004). The organisation of glomeruli within the AL in animals as diverse as insects (Galizia and Menzel, 2001; Laurent, 1999) and vertebrates (Mori *et al.*, 1999; Xu *et al.*, 2000) is such that a small number of glomeruli are combinatorial conditioned by a large number of odour stimuli (Vosshall *et al.*, 2000), and a distributed system of glomerular PNs is dynamically activated by a compound of multiple odours, and at the same time a specific PN actively represents multiple odour compounds (Christensen *et al.*, 2000; Christensen and White, 2000; Daly *et al.*, 2004; Kay and Laurent, 1999; Laurent, 1999; Laurent *et al.*, 2001; Lei *et al.*, 2002; Mori *et al.*, 1999; Sachse and Galizia, 2002). In vertebrates, the representation of a given odour in the OB is dependent on the experience with an odour (Bhalla and Bower, 1997; Fletcher and Wilson, 2003; Goldberg and Moulton, 1987; Kay and Laurent, 1999; Kendrick *et al.*, 1992; Sullivan *et al.*, 1989; Wilson and Sullivan, 1994; Wilson *et al.*, 1987), and learning-dependent changes in the insect AL have been observed using imaging techniques suggesting that experience-dependent structural changes might be happening in the insect AL (Faber *et al.*, 1999; Yu *et al.*, 2004) as well.

The studies on the experience-dependent plasticity in the AL of *M. sexta*, a model organism of which the anatomy and physiology of the AL are well known (Christensen *et al.*, 2000; Christensen and White, 2000; Daly *et al.*, 2004; Hildebrand and Shepherd, 1997; Kent *et al.*, 1987; Lei *et al.*, 2002; Rospars and Hildebrand, 2000; Tolbert and Hildebrand, 1981), reveal the relationships of behavioural measures of olfactory learning with neuro-physiological measures of odour-evoked ensemble responses in the AL before, during, and after olfactory conditioning (Daly *et al.*, 2001; Daly *et al.*, 2004; Daly and Smith, 2000). As in other insects, an olfactory stimulus followed by food resulted in recruitment of neural units in the AL, and lack of food reinforcement withered the responsive neural units (behaviourally referred to as extinction). Repeated reinforcement always increased the recruitment of neural units. All these findings conclusively show that the insect ALs consist of the synaptic neural circuits which are "plastic" in the sense that structural changes in neural networks in the AL occur dynamically during learning. The output pathways from the AL (PNs) relay dynamic signals which are odour-dependent patterns of inhibition followed by excitation (Daly *et al.*, 2004). The odour-driven responses within the AL are mediated by

correlated input from a different sensory modality, as in the case of a gustatory input, through feedback loops; for example, monoaminergic neurons in both *A. mellifera* (Hammer, 1993; Hammer and Menzel, 1995, 1998) and *M. sexta* (Kent *et al.*, 1987) exist in the AL providing signals from other brain areas. In honeybees, for example, a modulatory neuron VUMmx1 releases octopamine (Hammer and Menzel, 1995, 1998) when activated by sucrose. The AL plasticity may have many different roles including magnification of olfactory signals (Linster and Smith, 1997) and formation of the short-term olfactory memory (Faber *et al.*, 1999; Yu *et al.*, 2004). This highlights the need to understand at what point behaviour is altered temporally as sensory input changes. For example, in the Wasp Hound, when parasitic wasps are repeatedly exposed to an odour without food reinforcement, the association between the odour and food is extinguished. Extinction can be reversed with positive reinforcement at appropriate intervals.

Molecular biology of olfactory system

The expression of odourant receptor molecules in ORNs are finely tuned to a specific subset of odour molecules in the environment (Buck and Axel, 1991; Malnic *et al.*, 1999). The olfactory map, a spatial map of the distinct locations for specific odour signals within the AL, is organized according to the type of odourant receptor a particular ORN expresses.

A family of candidate genes, the Or genes, was discovered in *Drosophila* in 1999 (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). These genes control the expression of G protein-coupled receptors (GPCRs) as in the case of mammals (Buck and Axel, 1991) and in the nematode *Caenorhabditis elegans* (Troemel *et al.*, 1995). GPCRs have seven transmembrane domains and are expressed by a diverse range of gene sequences containing 62 members dispersed throughout the genome in small clusters (Clyne *et al.*, 1999; Gao and Chess, 1999; Robertson *et al.*, 2003; Vosshall *et al.*, 1999) which includes two genes that are alternatively spliced. Humans, mice and mosquitoes have approximately 350, 1,000, and 80 functional Or genes (Godfrey *et al.*, 2004; Hill *et al.*, 2002; Malnic *et al.*, 2004; Zhang and Firestein, 2002) respectively, but it is not clear if the number of Or genes are related to odour specificity in olfactory systems. The highly diverse gustatory receptor (Gr) gene family of *Drosophila* consists of 60 genes that encode 68 proteins through alternative splicing (Clyne *et al.*, 2000; Robertson *et al.*, 2003); many of them are up-or-down regulated in gustatory organs (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001); some work as taste receptors (Chyb *et al.* 2003; Dahanukar and Foster, 2001; Ueno *et al.*, 2001); and some are pheromone receptors (Bray and Amrein, 2003). Gr genes are also expressed in the antennae (Scott *et al.*, 2001) and tarsi (Ishimoto and Tanimura, 2004) of *Drosophila* and most probably a majority of insects.

Most of the *Drosophila* antennal odourant receptors, each of which has a unique odour spectrum despite being activated by common ligands, have now been mapped to the ORNs from which they are derived (Hallem *et al.*, 2004), and the complete odour spectrum of an ORN can be understood in terms of the odour spectra of the receptors mapped to that particular ORN. Furthermore, the spontaneous firing rate, response dynamics and signaling mode (excitation or inhibition) of the ORN (Hallem

et al., 2004) are also determined by the receptors providing a versatile functionality for each ORN.

As mentioned earlier, approximately 1320 ORNs of the antenna and maxillary palp in *Drosophila* connect with approximately 43 glomeruli in the AL. As in mammals, the axons of ORNs expressing the same odorant receptors connect to only one glomerulus or to a few glomeruli, providing us with a spatial map of ORN projections. Therefore, the pathways of the odour receptors bind the physiological units together functionally in the insect olfactory system although these units are at different locations. In addition, different sets of PNs in the AL are activated by different odours and their responses depend on the odour spectra, signaling mode and response dynamics (Wilson *et al.*, 2004) as in the case of ORNs. The activation of a PN is dependent on its pre-synaptic ORNs (Wang *et al.*, 2003). Wilson *et al.* (2004) suggested that PN output was dependent not only by ORN inputs but also on lateral inputs within the AL (Wilson *et al.*, 2004). Several genes involved in olfactory learning in *Drosophila* encode some of the components of the cAMP signaling pathway; the adenylyl cyclase encoded by the rutabaga (*rut*) gene; the cAMP phosphodiesterase encoded by the dunce (*dnc*) gene; the cAMP dependent protein kinase (PKA); the predicted product of the amnesiac (*amn*) gene; and the transcription factor cAMP-response element binding protein (CREB) (Davis, 2005; McGuire *et al.*, 2005).

Computer modeling of olfactory systems

Computational, mathematical and statistical modeling of various functional aspects of insect olfactory systems has increasingly been reported during the last decade. Getz (1991) developed a preliminary neural network for processing odour stimuli which can learn and identify the quality of an input vector or extract information from a sequence of correlated input vectors. Input vectors can be a sample of time varying olfactory stimuli. A discrete time content-addressable memory (CAM) module was developed to satisfy the Hopfield equations (Getz, 1991) with the addition of a unit time delay feedback, which improved the convergence properties of the network and was used to control a switch which activated the learning or template formation process when the input was “unknown”. The network based on CAM had dynamics embedded within a sniff cycle which included a larger time delay that was also controlled the template formation switch. In addition, this time delay modified the input into the CAM module so that the more dominant of two mingling odours or an odour increasing against a background of odours was identified. The network was evaluated using Monte Carlo simulations and it was concluded that a Hopfield type CAM may not be suitable for simulating an olfactory system; however, this pioneering work showed that artificial neural networks, which have multitudes of learning strategies embedded in the network, could be used to model the olfactory systems and their functions.

One of the earliest attempts to model olfactory functions was by Rossokhin and Tsitolovsky (1997) who focused on information processing by neurons. Biochemical reactions that were hypothesised to be controlling the properties of the excitable membranes in the nerve cells were modeled by a set of first order differential equations for chemical reaction kinetics by taking the effect of regulation of the properties of sodium channels into account. The neuron's electrical activity parameters occurring

during learning associated with its excitability were simulated, and they showed that the neuronal model exhibited different excitability after the learning procedure relative to the different input signals corresponding to the experimental data.

Pearce *et al.* (2001) modeled the efficiency of odour stimulus encoding within the early stages of an artificial olfactory system using a spiking neuronal model driven by fluorescent microbead chemosensors. The specific objective of the modeling study was to investigate how a rate-coding scheme compared to the direct transmission of graded potentials in terms of the accuracy of the estimate that an ideal observer may make about the stimulus. Their results showed how the charging time-constants of the first stages of neuronal information processing within the OB directly affected the reconstruction of the stimulus.

In an interesting study, Chang *et al.* (1998) developed a general connectionist model for an olfactory system by modeling the dynamical behaviour of each node (neural ensemble) by a second-order ordinary differential equation (ODE) followed by an asymmetric sigmoidal function, with which they modeled the aggregate activity of neurons in terms of system parameters and stimuli from an outside environment. The general connectionist model was used to simulate a mammalian olfactory system having modifiable synaptic connections and spatio-temporal interactions among neural ensembles. They developed a parameter optimization algorithm as an integral component of the model.

Ikeno *et al.* (1999) developed a model of the mushroom body of insects consisting of Kenyon cells based on the ionic currents in the isolated Kenyon cell somata in honey bees as measured by the whole-cell recording method. A rapidly activating and inactivating A-type potassium current, a calcium-activated potassium current and a delayed rectifier-type potassium current, and several types of inward currents were modeled by using Hodgkin/Huxley-type equations. They reconstructed the voltage responses of isolated Kenyon cell based on these mathematical models.

Christensen *et al.* (2001) developed a detailed multi-compartmental model of single local inter-neurons in the AL of the sphinx moth, *Manduca sexta*, using morphometric data from confocal-microscopic images, to study how the complex geometry of local neurons may affect signaling in the AL. Simulations clearly revealed a directionality in the neurons that impeded the propagation of injected currents from the sub-micron-diameter glomerular dendrites toward the much larger-diameter integrating segment in the coarse neuropils. They showed that the background activity typically recorded from LNs *in vivo* could influence synaptic integration and spike transformation in the local neurons. The modeling study supported the experimental findings suggesting that spiking inhibitory local neurons in the AL can operate as multifunctional units under different odour spectra. At low odour intensities, the neurons process mostly intra-glomerular signals; at high odour intensities the same neurons fired overshooting action potentials, resulting in the spread of inhibition globally across the AL. They concluded that the modulation of the passive and active properties of neurons were a deciding factor in defining the multi-glomerular odour representations in the insect brain. Getz *et al.* (1999) also developed a model for the olfactory coding within the insect AL using neural networks to investigate how synaptic strengths, feedback circuits and the steepness of neural activation functions influenced the formation of olfactory code in neurons within the AL. They reported that these factors were important in discriminating the dispersed odour spectra. Rospars *et al.* (2001) measured the spike

frequency of olfactory receptor cells in response to different odourants experimentally and developed the concentration-response curves which were accounted for by a model of the receptor cell they developed. This model, consisting of three main equations, suggested that most often the variability in sensitivity was due to the variability of odourant receptor binding characteristics.

Gu *et al.* (2007) developed a cross-scale dynamical neural network model to simulate the presentation, amplification and discrimination of host plant odours and sex pheromones to understand the dependence of dynamics of the olfactory maps in the AL on glomerular morphology. They used stochastic dynamical approaches to amplify weak signals and to discriminate odour signals. They used the neural network model to investigate arborizing patterns of the projection neurons (PNs) and timing patterns of the neuronal spiking activity.

There are a significant number of other studies which are rule- or neural network based utilizing similar methodologies of those already discussed (Av-Ron and Rospars, 1995; Chang and Freeman, 1996; Eisenberg *et al.*, 1989; Freeman *et al.*, 1988; French *et al.*, 2006; Getz and Lutz, 1999; Gu and Liljenström, 2007; Ikeno and Usui, 1999; Kaiser *et al.*, 2003; Kanzaki, 1996; Lei *et al.*, 2004; Ma and Krings, 2009; Patterson *et al.*, 2008; Quenet *et al.*, 2002; Rospars *et al.*, 2007; Snopok and Kruglenko, 2002; Webb, 2004); however, to understand insect olfactory systems in the regime of behaviours which are not measurable or observable, we need to develop phenomenological models based on the molecular biology of the olfactory systems so far discovered. Computer models that predict behaviours based on sensory input would need to simulate all the processes from sensory stimuli acquisition to bodily movements of the insect. Models based on actual neurophysiological and genetic processes would not only provide an avenue for understanding mechanisms of odourant conditioning, but also provide insight into how insects and other animals process olfactory signals. Such models could then be used to predict odourant properties based on behavioural observations (Rains *et al.*, 2008) and used as an enhancement to whole-organism programmable sensors.

Genetic breeding

Selective breeding is a traditional approach which allows researchers to sift out desired characteristic combinations in animals or plants without the expense associated with modern molecular techniques. However, depending on the organism of interest, such an approach can be extremely time consuming and expensive. Some organisms require specific environmental parameters while others produce relatively few offspring per generation.

An advantage of using selective breeding with invertebrates, such as insects, is that many species have a short generation cycle allowing for multiple life cycles throughout a year. And, costs associated with colony maintenance are less when compared with managing canines or other vertebrates. For example, in some instances, a cage and a little chicken feed is all that is needed to mass produce an insect (Tomberlin *et al.* 2002). Consequently, phenotypic traits can be isolated in resulting progeny rather quickly in comparison to canines or other vertebrate programs. Selective breeding for certain arthropods, such as the honeybee and silk moth, *Bombyx mori* (Lepidoptera: Bombycidae), proved fruitful resulting in greater efficiency in their management and the production of their associated products. However, repeated inbreeding has resulted

in the frequency of recessive traits increasing and producing various debilities. For example, the silk moth has been maintained in captivity for producing silk for well over 5,000 years. However, selective breeding for higher quality silk has resulted in various levels of susceptibility to nucleopolyhedroviruses (Ribeiro *et al.*, 2009).

In contrast, other selective breeding has resulted in greater fitness. Breeding programs for the honeybee have been successful in selecting for greater disease resistance (Evans *et al.*, 2006). Additionally, the simplicity of backcrossing allows for fine-scale genetic mapping and the isolation of behavioral trait-specific genetic regions (Oldroyd and Thompson, 2006). Recent evidence has determined genetic differences in the ability of arthropods to discriminate between target and non-target volatiles (Ferguson *et al.*, 2001). In fact, selection for greater discrimination performance can be achieved through only one generation (Ferguson *et al.*, 2001). Using selective breeding with insects still runs the risk of less desired recessive traits being expressed, and it can be difficult to formulate the right combination and level of expression of physical traits desired in the progeny. In regards to the honeybee, the negative effects of inbreeding are severe due to the genetic load introduced by the sex locus (Oldroyd and Thompson, 2006). Thus, selective breeding is still a gamble with each cross between genetic lines which can be frustrating and time consuming.

Deciphering the genetic mechanisms at work in sensory systems could lead to techniques for manipulating and enhancing the use of arthropods as biological sensors. We provide below examples of manipulations of the olfactory (smell) system in invertebrates which have led to refined sensing in the host organism.

The current insects used as biological sensors are diurnal. Responses to stimuli during non-active hours (i.e. night) result in declined response to external stimuli. Therefore, their use is ultimately limited by day length or light intensity. However, recent efforts have determined that it is possible to develop mutant strains that operate under dark conditions. Cheng and Nash (2008) determined that *D. melanogaster* bearing a mutation in the *inaF* gene, which is responsible for normal trp function (Li *et al.*, 1999) exhibited normal detection ability of the anesthetic halothane under dark conditions; however, once placed in ambient light the sensitivity decreased. Furthermore, Cheng and Nash (2008) determined that mutations in one gene resulted in greater sensitivity under ambient light. Therefore, increased sensitivity through genetic manipulations is a possible avenue in enhancing biosensor sensitivity and utility.

Insects are able to detect and respond to a variety of volatile compounds. Consequently, considerable time and resources are invested in conditioning insects to specific odourants of interest. Another hurdle with whole-insect programmable sensors is the reduction in response to target odourants by the conditioned insect over time (previously discussed extinction). Presently, conditioned arthropods, such as *M. croceipes*, are limited in the time they can be used as their response to target chemicals is reduced after repeated exposure. Behavioural responses can be re-established after re-conditioning (Tertuliano *et al.*, 2004) and can increase the length of time the wasps can be used as a sensor. However, it is possible that these insects could be genetically modified to enhance the behavioural response to select compounds. Therefore, conditioning could result in better responses for longer periods of time, or potentially for a permanent period of time of extinction could also be turned off at the genetic level. Morgan *et al.* (1988) determined that a variety of volatiles detected by a wild strain and three mutant strains of *C. elegans* varied. In some instances the mutant

strains were 30% more sensitive to select volatiles than the wild strain. Therefore, it is conceivable that insects used for biosensors could be modified to be more sensitive and specific in regards to what they detect and respond. In addition, the strength of response found in either EAG signals, cardiac response, or changes in observable behaviour, could also be manipulated through genetic engineering to strengthen response to select odourants without associative learning (hard-wired).

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