

Insect – host interactions

Signals, senses, and selection behaviour

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Mister Rector, ladies and gentlemen,

The large majority of insect species escape our every-day attention with only few notable exceptions: the colourful butterflies and the buzzing bees, houseflies and mosquitoes. Entomologists are a special breed among biologists, intrigued as they are by these small organisms and much aware of the extraordinary richness of the insect fauna in terrestrial ecosystems. The number of insects and other terrestrial arthropod species is huge relative to other animal taxa (upto 100.000 specimens per m² of soil is no exception; Paoletti *et al.*, 2007). More important than their diversity and numerical abundance, insects fulfil vital functions in plant reproduction in natural and agricultural ecosystems, in natural control of organisms harmful to mankind and in decomposition of organic matter (Schoonhoven *et al.*, 2005; Chapman, 2013). The number of insect species dwelling on Earth has been estimated to be at least 5.000.000 (Grimaldi and Engel, 2005). Approximately half of the 1.000.000 insect species taxonomically described depend on the photosynthetic capacity of plants to provide the nutrients they need to grow, develop and reproduce; these 500.000 plant-feeding species are collectively called herbivores; if we assume that also among the 4.000.000 insect species yet to be discovered 50% is herbivorous, the number of herbivorous insect species would be 2.500.000. The other half either feed on other animals, including the large guilds of predators and parasitoids, or on decaying organic material. The number of higher plant species currently described is ca. 300.000. Knowing these numbers one might conclude that the study of insect – plant interactions is an insurmountable task to undertake, because the number of potential interactions is 750 billion. Fortunately evolution has come to the rescue of the entomologist intrigued by insect-plant interactions. The evolution of insect-plant interactions is ongoing since ca. 350 million years and has resulted in the high degree of host-plant specialism that we currently observe and that is the major biological feature of these interactions (Figure 1).

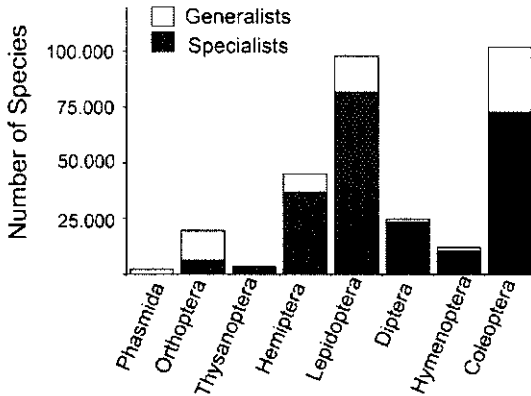


Figure 1. Numbers of specialist and generalist plant-feeding insects in the eight most speciose insect orders. Averaged over the orders, over 80% of plant-feeding insects are specialised feeders.

Over 80% of plant-feeding insects are highly specialised feeders: they confine their parasitic life style to plant species belonging to a single family of plants and in the large majority of cases even to a single plant species. An example of the latter is the butterfly featuring on the cover, the brimstone (*Gonepteryx rhamni* L.), a member of the family Pieridae; this species probably is the archetype for the generic English name 'butterflies' for the order Lepidoptera, because it is seen flying very early in spring in western Europe, the males having a butter-yellow colour. The brimstone is associated with only two host plant species in the same genus, the alder and common buckthorns (*Rhamnus frangula* L. and *R. cathartica* L.; Rhamnaceae). On the other extreme of the discontinuous scale of specialisation are so-called generalist or polyphagous plant-feeding insects that consume the tissues of several hundreds of phylogenetically unrelated plant species, among which our most important crop plants. Notorious examples of these are the *Helicoverpa* and *Spodoptera* armyworms, belonging to the family Noctuidae, that consistently rank among the top 10 of agricultural pest species world-wide. Of all living herbivorous insects less than 10% feeds on more than three different plant families. First I will discuss the various ways in which plant-feeding insects and their host plants interact.

Oviposition behaviour

I will discuss insect – plant interactions as they occur for three life stages of a butterfly and its host plants (Figure 2). Large Cabbage White butterflies (*Pieris brassicae* L.; Lepidoptera: Pieridae) overwinter as diapausing pupae and due to increasing daylength and rising temperatures in spring becomes active and eclose from their pupal case. After mating the female starts to search for host plants to lay

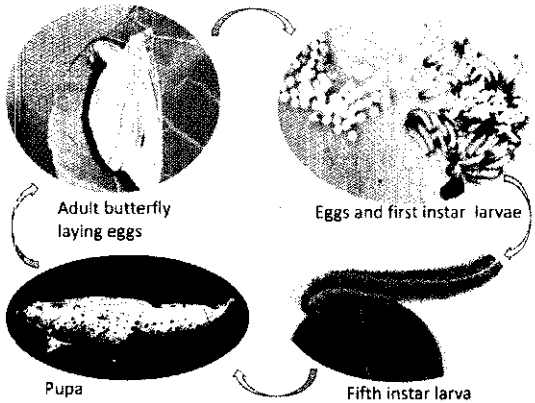


Figure 2. Life cycle of the Large Cabbage White butterfly *Pieris brassicae*. Top left: adult female, laying eggs at the underside of a cabbage leaf; top right: eggs and freshly hatched first instar caterpillars; bottom right: fifth (final) instar larva; bottom left: pupa (lateral view, ventral side up).

her eggs. Being a specialist species, the female needs to locate a plant that belongs to the family Brassicaceae, the mustards and cabbages. In accomplishing this, the female butterfly uses optical and olfactory signals associated with plants: the green colour of leaves, the display of flowers, and the volatile compounds that are released by the entire plant, including its roots. The female is attracted by green objects and detects in flight plant volatiles with olfactory neurons in hair-like structures called sensilla on the antennae (Figure 3).

The wall of an olfactory sensillum is perforated with minute holes, each of them measuring about 10 nanometer wide, over its entire surface. These pores allow the volatile molecules to enter the inside of the sensillum and after transport by odorant binding proteins (OBPs), to bind to receptor molecules in the membrane of the olfactory neuron. If the flux of molecules, that is the number of molecules binding receptor molecules per unit of time, is high enough, the detection threshold of the olfactory neuron is exceeded and the neuron will change the rate at which it generates action potentials that are conducted to the olfactory lobe of the butterfly brain (Figure 4). The pattern of electrophysiological activity received by the brain is assessed and is translated into a behavioural response along neural pathways that have begun to be mapped with respect to the antennal lobe and the mushroom bodies in the brain (Figure 4). Information from the hundreds of antennal sensilla harbouring several thousands of olfactory neurons is sorted into a much smaller number of processing units: in the antennal lobe of butterflies and moths between 60–80 glomerular structures can be discerned. Along neural processing pathways that as yet are largely unknown, coordinated motor output is generated that we observe

as behaviour. The olfactory detection of plant volatiles may elicit the female to alight on a plant, depending on the composition and concentration of the volatile blend.

For the Large Cabbage White female that is ready to lay eggs, it has been observed that she lands on more than a dozen plants before a cluster of eggs is deposited on one of them in a patch of host plants; this indicates that a notable investment in time and energy is made to select an oviposition site. Immediately after landing, she drums several times a second on the leaf surface with her forelegs. It is still debated if this causes mechanical damage to epidermal leaf cells, that would release dissolved compounds and to what extent sensory access is gained to polar compounds in the apolar matrix of the leaf epicuticle or in the epidermal or interior leaf cells. Scanning electron microscopy has not revealed apparent damage. The tarsi of the forelegs carry sensory hairs of ca. 30 micrometer long that have a single pore at their tip: these are taste sensilla (Figure 4). Every sensillum harbours four taste neurons. Such taste hairs occur on all six legs and the total number of tarsal taste neurons is ca. 2100, all of which project to the suboesophageal ganglion, a part of the brain situated below the foregut (Figure 4). A combination of phytochemical, behavioural and sensory approaches has been employed to analyse which chemical compounds produced by the plant act as signals affecting the selection behaviour of the female butterfly.

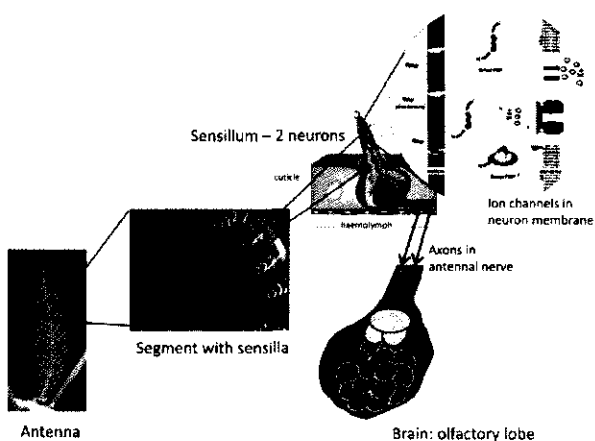


Figure 3. Antenna typical for a male moth (family Noctuidae), single antennal segment with rows of olfactory sensilla, schematic detail of single sensillum (longitudinal section). Circular inset: schematic detail of how pheromone molecules in the air outside the sensillum wall enter through minute pores (10 nanometer diameter) in the cuticle and bind to odorant-binding transport proteins (BmorPBP) in the lymph surrounding the dendritic double-layered lipid membrane of an olfactory neuron. Two ion channels for sodium ions (Na^+) and potassium ions (K^+) are shown in the membrane.

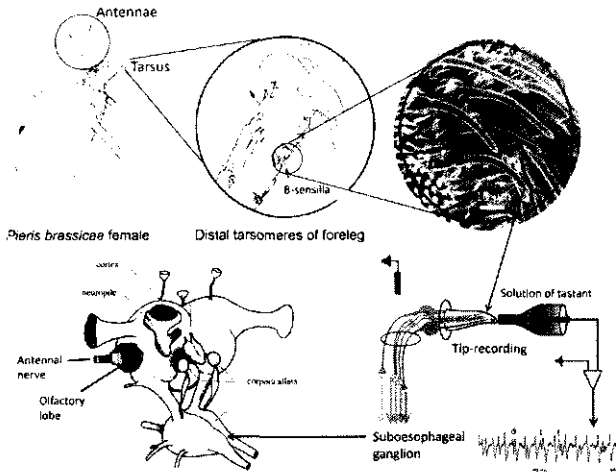


Figure 4. Top left: drawing of adult female of the Large Cabbage White butterfly *Pieris brassicae* (lateral view). Circular inset: drawing of two distal segments of the tarsus of the foreleg; top right inset: scanning electron micrograph of a cluster of trichoid taste sensilla; bottom right: schematic drawing of a single taste sensillum and the five neurons innervating the sensillum; a glass capillary is positioned close to the sensillum tip pore, used for stimulating the taste neurons with molecules in solution. At the bottom right, an exemplary recording of electrophysiological activity (a series of biphasic action potentials) generated by the taste neurons is shown. The axons of the five neurons run to the suboesophageal ganglion (bottom left), located ventrally in the head, the centre for processing taste information. The suboesophageal ganglion is connected to the brain with neural connectives running around the oesophagus of the foregut. Antennal nerve, olfactory lobe and the cortex and neuropile of the mushroom body (the seat of insect memory) are shown also.

The behavioural decision of acceptance or rejection for egg-laying depends in a surprisingly straight-forward way on the ratio between stimulatory and inhibitory inputs. This input – output approach is useful as it allows us to predict the behavioural effect of stimuli based on the electrophysiological activity they elicit and can be used for a pre-screening of active compounds. It reduces the outcome of processing in the brain to an arithmetic rule and makes no assumptions about the neural processes operating. Strong stimulatory input is generated by one of the four taste neurons (Figure 5) that responds to glucosinolates, a family of ca. 120 secondary plant compounds that are synthesised by species in the Brassicaceae, the favourite host-plant family of *P. brassicae* and other *Pieris* species. The glucosinolates are compounds that have a defensive function against plant-feeding animals, bacteria and fungi, except for a small number of specialised species that have evolved different biochemical mechanisms to detoxify these molecules (Hopkins *et al.* 2009).

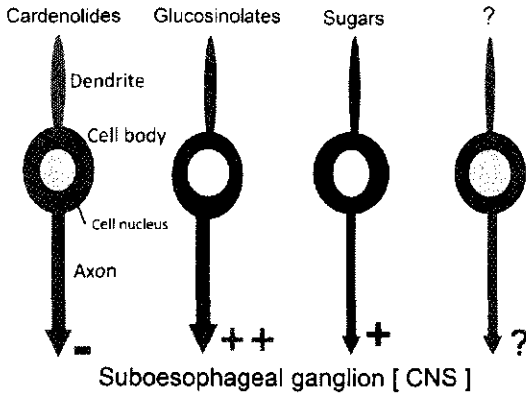


Figure 5. Schematic drawing of four taste neurons housed in one taste sensillum on the tarsus of a butterfly (cf. Figure 4). In the top the class of plant compounds to which each neuron responds is indicated at the tip of the dendrites; for one of the neurons the proper ligands are unknown. The axons run into the suboesophageal ganglion of the central nervous system (CNS; cf. Fig. 4). The effect of neuron activity on butterfly oviposition or feeding behaviour is indicated: - : inhibitory effect; + and ++ : stimulatory effects.

Different *Pieris* species differ in their sensitivity profiles to glucosinolates and also at the sub-species level sensitivity profiles differ significantly (Du *et al.*, 1995). Strong inhibitory input is provided by a second type of taste neuron in the tarsal sensilla. This neuron responds to cardenolides, a small family of steroidal compounds that only occur in a few genera of the Brassicaceae, such as wild mustards (*Erysimum* spp.) that are not accepted for oviposition despite producing glucosinolates (Du *et al.*, 1995). This allows the female to avoid exposing her offspring, the larvae hatching from the eggs, to the toxic cardenolides. The taste system of the larvae, consisting of only 120 neurons, has similar neuron types that detect glucosinolates and cardenolides (Van Loon and Schoonhoven, 1999; Schoonhoven and van Loon, 2002). The neural encoding underlying this discriminatory behaviour combines labelled-line and across-fibre coding principles (van Loon *et al.*, 2008). The butterfly taste system is also involved in associative learning between leaf colour and stimulatory or inhibitory taste cues (Smallegange *et al.*, 2005).

The integration of information gathered by its visual, olfactory and taste systems enables the insect to make more subtle decisions than acceptance or rejection. Within the range of plants that are acceptable in a no-choice situation, female insects show a preference hierarchy when given a choice and also within a plant species or cultivar females display selectiveness. We know that females respond to differences in plant physiological condition related to plant nutrient status, photosynthetic capacity, plant hormone status, past feeding damage and presence of conspecific eggs.

However, which phytochemical differences are detected by the butterfly chemosensory systems to guide behavioural selection defies our current understanding. Considerable phenotypic plasticity has been found for insect taste neurons as a result of exposure to different diets (Schoonhoven and van Loon, 2002; Zhou *et al.*, 2009; 2010). As the complexity of the non-volatile metabolome of the plant escapes exhaustive chemical characterisation, the way forward to understand how the taste system encodes the chemical profile of the plant is to offer the complex natural stimulus itself (Van Loon *et al.*, 2008). Renewed attention for insect taste is warranted.

Plant responses to insect eggs: the initial phase in insect – plant interactions

The next phase in the insect-host-plant interaction is that of egg contact with the plant. For several insect species the deposition of chemical markers on the substrate on or in which eggs are laid was known (Nufio and Papaj, 2001) and indications for the use of host-marking pheromones that deter oviposition by females visiting such occupied substrates have also been found for cabbage white butterflies (Schoonhoven, 1990). A long search for the chemical nature of the active molecules yielded extremely effective deterrent compounds present in the accessory glands of the female reproductive system (Blaakmeer *et al.*, 1994a). Follow-up experiments, however, on the mobility and stability of these molecules in the leaf made their role unlikely. Since then it has become clear that despite the apparently harmless nature of the eggs, their presence is noted by the plant and it is the plant response to eggs that constitutes the signal to females (Blaakmeer *et al.*, 1994b; Fatouros, 2012; Little *et al.*, 2007). Within a few hours, changes in the expression of genes involved in plant defence take place, leading to an early alert of imminent damage that will only commence several days later, the time needed for the embryo to develop to a neonate caterpillar hatching from the egg (Fatouros *et al.*, 2008; Gouhier-Darimont *et al.*, 2013; Hilker and Meiners, 2010). The eggs are meticulously placed on the plant surface using a cement-like substance produced by the accessory glands. Little is known about the nature of putative elicitors associated with insect eggs that induce the enhanced expression of genes in the salicylic acid hormonal pathway of signal transduction in the plant: are these contained in the secretions from the accessory glands or is there diffusion of compounds from the egg into the leaf? In the interactions between two *Pieris* butterfly species and black mustard (*Brassica nigra* L.) and other wild *Brassica* species a hypersensitive response to the eggs is observed that is rare in insect-plant interactions, however, well-known from microbial pathogen - plant interactions. It is of interest to identify the origin and chemical nature of the elicitors and the receptors of the plant that mediate the hypersensitive response to compare these with those involved in such responses to fungal pathogens. In the egg

phase we have identified a role of an anti-sex pheromone (benzylcyanide) that the male transfers to the female during mating. When this compound is applied on the leaf surface at natural concentrations, leaf surface chemistry changes so as to arrest minute egg parasitoid wasps of the family Trichogrammatidae, important biological control agents that are world-wide successfully employed in agriculture. These studies on interactions preceding and following oviposition, the behaviour crucial for insect reproduction, reveal the intricacy of the interactions mediated by chemical signals.

Whereas it has been common practice in insect-plant studies that the egg phase was skipped and actively feeding caterpillars were put on the plant to initiate the interaction, we recently discovered unprecedented species-specific effects of eggs by increasing plant defence against caterpillars (Pashalidou *et al.*, 2013). Studies of the interactions occurring in the egg phase that started in laboratory and greenhouse have been expanded to studies in the field and have revealed a novel plant defence strategy of 'reproductive escape', by which black mustard plants accelerate seed development even before the young caterpillars reach the flowers (Lucas-Barbosa *et al.*, 2013; Pashalidou *et al.*, 2013). Paying explicit attention to the initial phase of the insect-plant interaction has brought us a number of important new insights and have yielded new challenging questions.

Plant responses to cell damage: induced direct defence

We have arrived at the moment in the life cycle of the butterfly at which the neonate caterpillar crawls from the egg shell (Figure 2), consumes it and then starts its voracious leaf feeding. The neonate caterpillar has a body mass of 0.2 milligrams; 15 days later it has grown to a pupa weighing 500 milligrams, a factor 2.500 increase: no other animal organism is growing this fast. During this growth process the caterpillar puts through plant biomass that amounts up to five times its own body mass per day. As soon as the caterpillar starts chewing the leaf, plant cells are ruptured, leading to wound reactions. These wound reactions involve a dynamic cascade of molecular responses in which the expression of hundreds of genes is changing, as has been demonstrated by micro-array studies in several insect-plant interactions (Reymond *et al.*, 2004, Broekgaarden *et al.*, 2007). To trigger a full-blown plant response, the combination of mechanical damage done by the chewing mouthparts and the recognition of molecules released by the insect, conveniently called 'spit factors', is required. Molecules in the secretions deposited by the insect's mouthparts onto the wounded plant tissue act as elicitors of the plant's reaction. The chemical nature of the elicitors identified thus far is remarkably diverse: macromolecules such as enzymes, peptides and small molecules such as fatty acid-amino acid conjugates or benzylcyanide (Maffei *et al.*, 2012). The biosynthetic origin of these elicitors is unknown in most cases: they may be secreted by salivary glands, by other glands

associated with mouthparts or may come with regurgitation of digestive fluids in the insect's foregut that contain plant compounds and their breakdown products and likely also compounds secreted by microbes living in the gut; there is a chemical mini-universe to be unravelled here. An important aspect of these induced plant responses is that they do not only occur locally at the site of damage but are translocated systemically throughout the plant. In this way, leaves or fruits that have not yet been damaged display increased resistance.

Understanding the signal-transduction pathways that occur in the plant is currently very actively being pursued in insect-plant research. At least three different hormone-mediated signalling pathways play a role in response to insect feeding: the jasmonic acid or octadecanoid pathway is activated in response to chewing insects, the salicylic acid pathway and the ethylene pathways are more strongly activated by piercing-sucking insects such as aphids and whiteflies. More recently involvement of other plant hormones have been found to act as modulators of jasmonate-mediated responses (abscisic acid, auxin, gibberellins) (Pieterse *et al.*, 2012). These pathways do not function independently from each other but are intertwined through cross-talk. An example of negative cross-talk is that prior aphid infestation of a cabbage plant leads to induced susceptibility to caterpillars. Aphid-infested plants have 10 times lower jasmonic acid concentrations (Soler *et al.*, 2012).

To unravel and manipulate the complex signalling network that plants have evolved to resist insect attack we have adopted the model plant *Arabidopsis thaliana* (L.) Heynh., a small weedy member of the Brassicaceae family and a suitable host plant for *Pieris* species as well as for another specialist herbivore, the Diamondback moth (*Plutella xylostella* L.; Lepidoptera: Yponomeutidae), a specialist herbivore yet a top-10 agricultural pest species. *Arabidopsis thaliana* was the first plant species for which the full genomic sequence became available and a large community of plant scientists freely exchange a large collection of mutants modified in all kinds of traits. Gene expression studies on *Arabidopsis* – insect interactions have revealed the complex genetic regulation of the responses to insect feeding and have given indications which metabolic pathways are up- or downregulated. We have focussed on expression of key regulatory genes in the jasmonic and salicylic acid pathways and the consequences of changes in expression of these genes for the biosynthesis of glucosinolates and of herbivore-induced plant volatiles, including volatile breakdown products of glucosinolates. In response to caterpillar feeding the biosynthesis of the sub-group of indole-glucosinolates is up-regulated, an observation that has also been made for *Brassica* species (Gols *et al.*, 2008; Kos *et al.*, 2012a). Increased tissue concentrations of glucosinolates have little consequence for the specialist *Pieris* and *Plutella* caterpillars but do negatively affect growth of the generalist *Mamestra* caterpillars (Poelman *et al.*, 2008). The specialists possess

specialised detoxification routes that divert the breakdown of the toxic glucosinolates to less toxic compounds (Hopkins *et al.*, 2009). In studies of ecotypes that differed in glucosinolate levels, performance of a specialised aphid was actually higher on an ecotype with higher total glucosinolates contents but larvae of a hoverfly (Diptera: Syrphidae), a common predator of aphids, had reduced performance (Kos *et al.*, 2012b). This is an illustration of the commonly found phenomenon that specialist herbivorous insects sequester secondary plant metabolites to exploit them for their own defence (Figure 6).

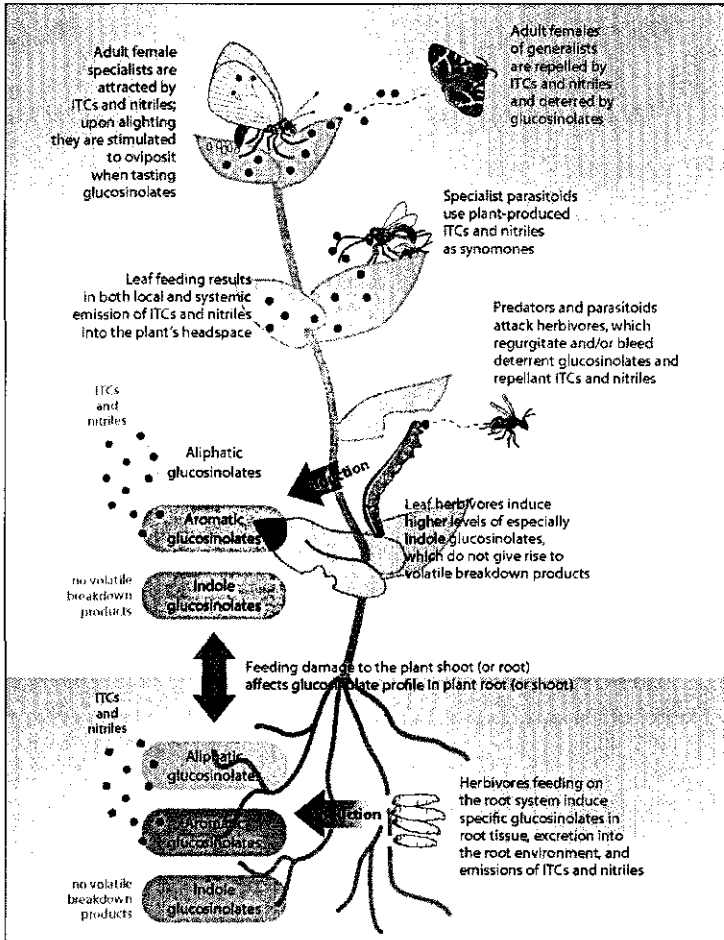


Figure 6. Illustration of the induction by plant-feeding insects of the three major groups of glucosinolates, defensive plant metabolites characteristic for the family Brassicaceae, the emission of their volatile breakdown products, isothiocyanates (ITCs) and nitriles, and their effects on behaviour of herbivorous and carnivorous insects. Taken from Hopkins *et al.* (2009).

Glucosinolates function in direct plant defence but have low or negligible effectiveness against specialists. This does not mean, however, that it is impossible to find resistance to specialist herbivorous insects. Recent experiments on the *Arabidopsis* – *Pieris brassicae* interaction demonstrated that overexpression of the transcription factor MYB75 resulted in re-channelling of flavonoid biosynthesis that revealed the importance of a flavonoid glucoside in resistance (Onkokesung *et al.*, unpubl.). In a recently started research programme natural genetic variation in resistance of 360 accessions of *Arabidopsis thaliana* to the combination of herbivory, fungal infection and drought is screened with the aim to identify putative genes and signalling pathways leading to increased resistance to such combined stresses.

Plant responses to cell damage: induced indirect defence mediated by plant volatiles

Responses of plants to cell damage also involves the release of volatiles into the atmosphere. Such volatiles may be exploited as signals through olfactory detection by herbivores and other organisms. Much information has become available on the changes in volatile emission that occur after tissue damage. Both qualitative and quantitative changes in the composition of the volatile blend are found and occur with different time courses depending on the biosynthetic origin of the compounds: (1) six-carbon alcohols, aldehydes and acetates known as green leaf volatiles; (2) mono-, sesqui- and homoterpenes and (3) benzenoid compounds such as methylsalicylate. Natural enemies of herbivores respond sensitively to herbivore-induced plant volatiles and orient themselves to the damaged plant. This phenomenon has become known as 'recruitment of bodyguards' by the plant, and is common in tritrophic interactions between plants, plant-feeding insects and their natural enemies. Little is known about the composition of the minimal volatile blend required to elicit the same degree of attraction of predators and parasitoids as exerted by an herbivore-damaged plant; identifying the relevant chemicals has turned out to be extremely difficult with only few exceptions (Dicke *et al.*, 2009).

Olfactory basis of host selection by parasitoids

The olfactory system of parasitoids is sensitive to a range of compounds resulting from different biosynthetic pathways as shown by on-line electroantennography linked to gas chromatography (Smid *et al.*, 2002). Hymenopteran parasitoids possess a special type of olfactory sensillum that is absent in Lepidoptera, the placoid sensillum, innervated by some 30 neurons that extend their dendrites in a cavity below the pore plate in the cuticle. The number of glomeruli in the antennal lobe of two *Cotesia* species is ca. 180, 2-3 times as many as the average number of glomeruli of Lepidoptera (Smid *et al.*, 2003). This suggests a higher diversity of response types in the antennal olfactory neuron population of the parasitoids than that of their hosts.

However, functional studies of olfactory neuron specificity and sensitivity in parasitoids are virtually absent in the literature.

Host selection behaviour of many parasitoid species shows a high degree of selectiveness: after a first rewarding experience of oviposition into the host or even contacting host-derived products such as frass or silk, the parasitoid female displays an increased preference based on associative learning of volatile cues that signal the presence of this host species. The female wasps learn the difference between volatile blends emitted by different plant species or cultivars attacked by the same host, different developmental stages of the host (Dicke and van Loon, 2000; Dicke *et al.*, 2009) or between unparasitised hosts and hosts that have been parasitised before and therefore may have become less suitable (Fatouros *et al.*, 2005). Closely related parasitoid species that differ in their degree of host specialisation acquire memory at different rates (Smid *et al.*, 2007).

From laboratory and greenhouse to the field

An important decision we took 10 years ago was to study tritrophic interactions under field conditions. In addition to cabbage (*Brassica oleracea* L.) we adopted black mustard (*Brassica nigra* Koch), an annual feral *Brassica* species. This fast-growing herb prompted us to include pollination biology and to address evolutionary mechanisms in multitrophic systems (Lankau *et al.*, 2008; Lucas-Barbosa *et al.*, 2013; Soler *et al.*, 2009). If we inspect cabbage or mustard plants growing in the field, a whole community of plant-feeding insects is found: other lepidopteran species, leaf-mining fly larvae, flea beetles and aphids; parasitoids and predators and flower visitors: plants are by default under multiple attack. A striking finding that came out of this field work is that herbivore attack early in the season has a strong influence on the community of insects that develops over the season (Poelman *et al.*, 2008; 2010).

Fundamentally different feeding modes in plant-feeding insects

Caterpillars, locusts, maggots and larvae and adults of beetles have biting-chewing mouthparts that tear off pieces of leaf and rupture plant cells. In contrast, aphids, whiteflies and other homopteran insects have piercing-sucking mouthparts and employ a completely different feeding mode that leaves plant cells intact (Figure 7). The very thin and flexible stylets, their diameter is a few micrometers, penetrate in between leaf cells and gelling saliva is secreted along the stylet pathway. The stylets are on their way to the vascular bundle of the plant's transport system and perform brief punctures through the membrane of cells without inflicting permanent injury. Femtoliter sap samples are transported to an interior taste organ located at the dorsal side of the pharynx. If the taste information is evaluated positively, the stylets move further until they reach the phloem, the plant tissue transporting the assimilates of photosynthesis. Once the membrane of a phloem sieve element has been punctured,

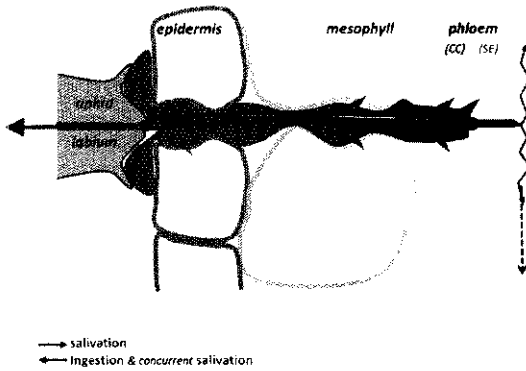


Figure 7. Aphid feeding. Overview of how aphids mouthparts move in between plant cells (lateral transverse view). The aphid labium rests on the leaf surface, gelling saliva (dark grey) has been secreted and the bundle of four stylets (black lines; diameter 2 micrometer) have been inserted in between epidermal and mesophyll cells and in between companion cells (CC) of the phloem and have reached a sieve element (SE) of the phloem, in which photosynthetic assimilates are transported. The aphid briefly punctured the membrane of every cell along its way to the phloem (dark grey spikes along the salivary track). When the stylet tips reach the phloem, secretion of watery saliva starts (two-pointed vertical arrows in the sieve element). The arrow pointing to left indicates the direction of ingestion of phloem sap into the aphid foregut, while concurrently secreting watery saliva to prevent clogging of the pores of the sieve plate (light grey stippled line). (Courtesy of Dr. W.F. Tjallingii).

a second type of 'watery' saliva is produced that contains effectors that suppress the wound reaction in the sieve element. The suppression is brought about by effector proteins that bind calcium ions, thus preventing the deposition of phloem proteins that would clog the pores in the sieve plate between phloem cells. Piercing-sucking insects cause increasing economic damage to food crops, an important reason being that they act as efficient vectors of a range of plant viruses and other plant pathogens. Both notoriously polyphagous species such as the green peach aphid *Myzus persicae* Sulzer and the tobacco whitefly *Bemisia tabaci* Gennadius and specialist species such as the black currant-lettuce aphid *Nasonovia ribisnigri* Mosely are evolving an increasing number of biotypes that are insensitive to resistance mechanisms that resulted from long-term breeding programs in a range of food crops. The quantities of saliva that piercing-sucking species inject into phloem elements are minute and it is a considerable but unavoidable challenge to clarify the function of salivary components in the insect-plant interaction and to identify the target molecules in the phloem. The information available thus far suggests that aphid saliva contains dozens of peptides and proteins, among which enzymes that degrade plant macromolecules. One way forward is to employ transcriptomic analysis of the salivary glands and RNA-based silencing techniques to elucidate the function of individual genes.

Future directions in insect-plant research

I have given you a condensed account of the intricate ways in which insects and plants interact in different phases of the insect life cycle. Attention in mechanistic research has shifted from studies of the sensory basis of behavioural selection by insects and its phytochemical basis to the molecular mechanisms operating in herbivore-induced responses of the plant. Recently renewed interest in the responses of the insect can be noted: how does it react behaviourally and metabolically to induced plant resistance and which selection mechanisms operate? What are the roles of elicitors and suppressors in insect saliva and which are the receptors in the plant? The effects of multiple herbivory in sequence or simultaneously need to be studied to gain a better understanding of the realistic conditions of multitrophic systems in nature. Which consequences does multiple herbivory have for plant resistance and what effects do we see for herbivore, parasitoid, predator and pollinator behaviour and performance? Major differences between biting-chewing and piercing-sucking herbivores and between generalist and specialist species are to be expected. How do plants respond to combinations of attackers i.e. insects, fungi and drought stress and can we find genetic variation for such combined stresses? The answers we find to these questions will find much-needed applications in plant breeding and biological control.

Mosquito – host interactions: chemosensory ecology

The interactions between blood-sucking mosquitoes and their animal and human hosts are evolutionary younger than those between insects and plants, however, they date back an estimated 100 million years, halfway the Cretaceous. Still today adult mosquitoes interact with plants as they feed on flower nectar as a vital source of carbohydrates; they likely evolved from piercing-sucking plant-feeding insects (Takken and Verhulst, 2013; Figure 8). The degree of host specificity observed in mosquito – host interactions is overall low compared to specialised insect-plant interactions, although some species are more anthropophilic than others and especially those species act as vectors of a range of infectious diseases, including malaria, dengue, West Nile and several others that show increases in incidence over the last decades. Among the primary signals that guide mosquitoes to their hosts is carbon dioxide exhaled by vertebrate hosts (Mboera *et al.*, 1997). Body temperature and humidity have also been demonstrated to elicit mosquito approach and landing; these stimuli are sensed by specialised sensilla on the antennae (Wang *et al.*, 2009). Since some 20 years it is firmly established that host-seeking by mosquitoes is to a large extent based on detection of host-produced odours.

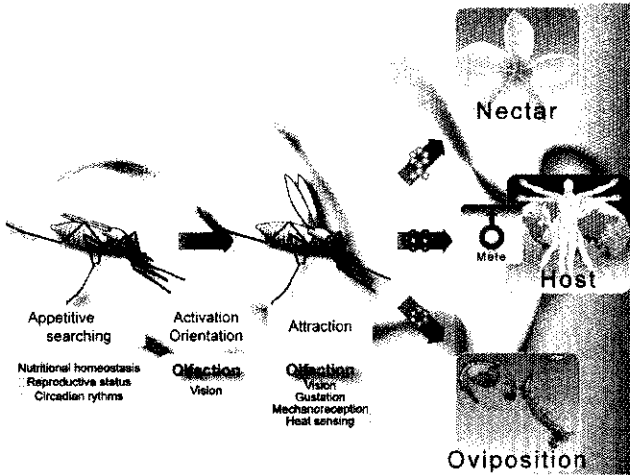


Figure 8. Mosquito behaviour is affected by volatile compounds released from flowers (sources of sugar-rich nectar), blood hosts (e.g. human beings), and aquatic oviposition sites. Three behavioural phases are indicated and the type of information utilised by female mosquitoes to find resources vital to reproduce. (Courtesy of Dr. L.J. Zwiebel).

Mosquitoes have olfactory sensilla on several appendages (Figure 9). Different from the relatively uniform morphology of the olfactory sensilla on the antenna of the Cabbage White butterfly discussed previously, the mosquito antenna has several

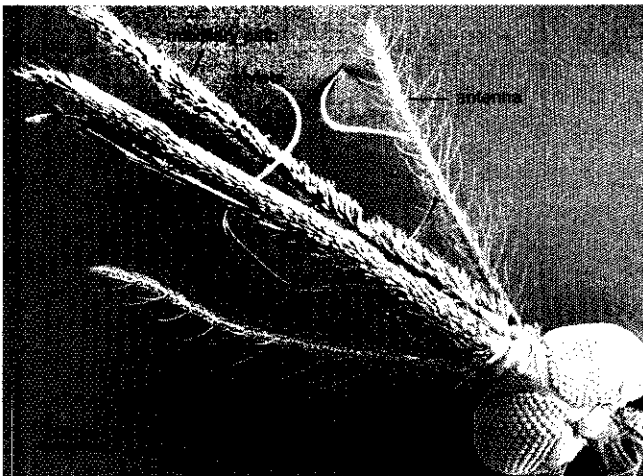


Figure 9. Scanning electron micrograph of mosquito head (bottom right corner) with appendages: antennae and mouthparts carrying sensilla for olfaction, taste, mechano-, thermo- and hygro-reception. (Courtesy of Dr. L.J. Zwiebel).

morphological types and subtypes, that also show different anatomy. It has been a major effort in our laboratory to make an electrophysiological inventory of the functional types associated with the morphological subtypes (Qiu *et al.*, 2006; Suer *et al.*, unpubl.). A panel of about 140 volatile compounds that have been reported from humans or animals and from the aquatic mosquito oviposition sites, have been tested for their effectiveness to elicit changes in electrophysiological activity. Thus far we have identified 25 response types based on the profile of activity resulting from exposure to these 140 compounds. This number comes close to the number of glomeruli found in the olfactory lobe of the mosquito brain (cf. Figure 4), suggesting that our inventory has come close to being complete. Several compounds in the panel did not elicit any response and have therefore been discarded for behavioural assays. The electrophysiological inventory was a necessary intermediary phase in a 'discovery pipeline' of behaviourally active compounds that we set up together with two colleagues in the United States, in the framework of a large 5-year programme funded by the Bill and Melinda Gates Foundation that also involved partners in Tanzania, The Gambia and Kenya. At one end of the pipeline over 250.000 organic molecules were subjected to high-throughput assays using heterologous expression of the malaria mosquito's olfactory receptor proteins in human kidney cells, frog oocytes or *Drosophila* antennae. The malaria mosquito has 79 of such olfactory receptor (OR) genes in its genome, of which 54 were found to be functional. Molecules that were strong ligands for one or more of these receptors in one of the heterologous assays were then put to the test in our laboratory by electrophysiological assays on single olfactory sensilla on the mosquito antenna. Only if activity above a set threshold was found in this last assay, it was subjected to behavioural assays that are much more laborious. The rationale behind this large effort was that any molecule that would impact on the mosquito's olfactory physiology, either of natural or entirely synthetic origin, might have potential for disrupting mosquito host-seeking behaviour. The number of candidate compounds that we identified along this pipeline has been surprisingly low, suggesting a high degree of tuning to naturally occurring ligands. Another explanation is the fact that the 'discovery pipeline' at the molecular level was entirely based on the OR-gene family whereas since then a novel olfactory receptor family (ionotropic receptor- or IR-family) has been revealed in insects (Benton *et al.*, 2009). Our electrophysiological work has protected us from completely missing out ligands of the IRs. In parallel we proceeded with the identification of volatile compounds released by human hosts that cause attraction of mosquitoes, which has turned out to be a challenging enterprise. One reason is the high number of volatiles that is released by human hosts, a total of ca. 350 compounds has been reported. Another cause is the difficulty to retrieve volatiles from the human body and to obtain these without

chemical contamination, that can cause high variability inherent to the individuality of human behaviour.

We have taken a variety of approaches combining volatile collection, electrophysiology and behavioural assays to tackle these problems, focussing on the most important vector of malaria in Africa, *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae). An important step was made when we discovered that fresh human sweat was much less attractive for female mosquitoes in search of a blood meal than sweat incubated at human skin temperature for a day (Meijerink *et al.*, 2000). Main components of incubated sweat were ammonia and lactic acid (Braks *et al.*, 2001). Based on odour collections from human volunteers, a range of carboxylic acids were found. We then combined ammonia, lactic acid and single carboxylic acids and systematically tested them at different concentrations in behavioural assays. In this way we arrived at a basic blend of three compounds, ammonia, lactic acid and tetradecanoic acid that showed a synergistic action and that we subsequently used as our basic blend upon which to build further in formulating an expanded attractive blend. Such blends have been evaluated in semi-field and in African villages and have shown a degree of attractiveness that competes with that of human beings (Mukabana *et al.*, 2012a,b). By now we have arrived at a blend of seven compounds, all of which are found in natural human emanations; leaving out any of these seven compounds results in significant loss of attractiveness. In parallel we followed a differential approach that is making use of the observation that human individuals differ significantly in attractiveness to malaria mosquitoes. Out of a population of 52 male individuals a group of seven showed significantly lower attractiveness than the seven most attractive individuals. This difference was associated with significant differences in the composition of skin bacteria (Verhulst *et al.*, 2010). These findings point to an added complexity of mosquito – host interactions: the role of skin microbiota. This in turn points to the involvement of the human immune system, in particular of variation in genes of the major histocompatibility complex (MHC) that have a role in determining the composition of skin microbiota (Verhulst *et al.*, 2013). Currently we are putting the highly attractive blends we developed to the test in villages in Kenya to find out if deployment of odour-baited traps contribute to reduce malaria infection rate. We are also developing a push-pull approach by combining traps baited with attractive blends (pull) with dispensers that release spatial repellents (push) and the first results are promising. Another challenge ahead is tailoring attractive blends for other malaria vector species. The odour-based strategy for disrupting the behaviour of these important disease vectors is one of several approaches being studied in our laboratory to impact mosquito populations that together must lead to integrated vector management.

Acknowledgements

Mr. Rector, near the end of this lecture I would like to thank the many people who have contributed in numerous ways to my scientific and personal development.

First I would like to thank my promotor Louis Schoonhoven whom I first met in 1975 at the Laboratory of Animal Physiology when I followed his classes in general and comparative physiology and ecophysiology. My second thesis was supervised by Wim van Muiswinkel of Cell Biology and Immunology. I am much indebted to both for shaping my interest in the functioning of the individual organism.

In 1981, when Louis Schoonhoven was invited to step up as Dean of the faculty he approached me if I was interested to keep the chemosensory research going in the Laboratory of Animal Physiology during a 4-year appointment. I gladly made use of this opportunity to work on a PhD-thesis. Louis, after your retirement in 1992, you involved me in writing a book on insect-plant biology, a challenging enterprise for which you did most of the hard work. It is gratifying that we are currently preparing the third edition of this book that has become well established as a course book in the field.

Freddy Tjallingii, you were my closest colleague in those early days of my scientific career at the Laboratory of Animal Physiology. We first met in 1975 when you taught practicals animal physiology. You supported me as a mentor and helped out with many technical matters. You organised the 'Kleine Club'-meetings, informal two-weekly gatherings of insect-plant workers of Entomology, the former Institute of Plant protection IPO and Animal Physiology. During these meetings my view of the insect-plant field was extended well beyond my own experimental work. I thank you for your continuous support and friendship.

Since 1989 until now I have been privileged to work at Entomology. Already in the first years of my appointment my new colleagues Marcel Dicke and Louise Vet involved me in making plans to adopt a common set of insect-plant and tritrophic interactions that we would study jointly, each of us taking approaches based on our own expertise and interest. We selected the interactions among brassicaceous plants, several species of Cabbage White Butterflies and their associated *Cotesia* parasitoids wasps that I have described in the foregoing. This explicitly joint effort has much stimulated the coherence of our group and has paid off very well in terms of productivity and funding. It has stimulated me to broaden my interest to behavioural ecology. For the chemistry of the signalling compounds we had a productive collaboration with Aede de Groot, Teris van Beek and Maarten Posthumus of the Laboratory of Organic Chemistry, that has recently been renewed for joint work on flavonoids.

Joop van Lenteren, you have strongly facilitated team-work in the group and I thank you for your constructive leadership and energetic commitment to guide the reorganisation of the laboratory in difficult times. Your advocacy for the adoption of biological control in crop protection and the quality of the basic ecological science on which this is based is a prime example of successful applied science.

Marcel Dicke, you head our group since 10 years in an excellent way. Your strong drive to practice high-quality science, your initiatives in acquiring funding and reaching out to the general public, your commitment to the cohesion within the group, your keen eye for synergism and for the well-being of staff and students stand out. I consider myself fortunate to have you as a colleague who generously shares his insights.

Willem Takken, you approached me 20 years ago to step into mosquito biology, a field that is both fascinating and of high societal relevance in the developing world and increasingly so in the western world. We have carried out several larger research programmes together and have made good progress in developing sustainable methods of manipulating mosquito behaviour. Your expertise in the biology of insect vectors is admirable and I thank you for involving me in your line of work.

Arnold van Huis, you are a strong advocate of the societal relevance of entomology. Apart from your work on crop protection in the tropics in which you integrated natural and social sciences, you showed vision by promoting the large-scale production of insects as a neglected source of nutrients, with potential to contribute to future food security. Thank you for asking me to join this promising expansion of our work.

Louise Vet, you have been instrumental in shaping the behavioural approaches in our joint work. Your contagious enthusiasm for biological science and your spontaneity are always refreshing. Although you stepped up as Director of the Netherlands Institute of Ecology 14 years ago, you kept the contacts with Entomology very much alive.

Hans Smid, you are not only a very pleasant roommate in Radix but are also at any time prepared to assist in overcoming technical or logistical challenges that present themselves, in a very skilful way, for which I am thankful. Together with Louise Vet you developed a productive research line on the molecular basis of memory formation in several parasitoid wasp species. I hope we will realise our plans to further explore the neural pathways involved in olfactory learning.

I thank my colleagues Maarten Jongtsma, Ben Vosman, Roeland Voorrips and Greet Steenhuis-Broers of Plant Research International for their pleasant collaboration.

Tiny van Boekel and Catriona Lakemond are our natural partners in expanding the research on insects as an alternative protein source for feed and food; thank you for sharing your expertise and your commitment.

All of you present here whom I did not mention by name, rest assured that I have appreciated the various ways in which you have been helpful and making me enjoy my work.

One of the activities I enjoy most in my work is the interaction with students, both undergraduates and graduates. I perceive it as a major responsibility to provide personalised supervision and to coach PhD students to become independent and critical scientists. Also the intensive exchange with the dedicated post-doctoral colleagues who joined and join our group has played an important role in my personal development.

Wageningen University and Research Centre is the largest centre of biological research in our country. It has been and will continue to be influential in improving the sustainability of agriculture, the conservation of natural resources and the protection of environmental quality. Its influence has reached out all over the world. Both Dutch and international students receive inspirational training that provides a solid basis on which to build their professional career.

The easy accessibility and collaborations that come about naturally are characteristic for the scientific atmosphere in Wageningen and are to be cherished. Being a Wageningen biologist is practising curiosity-driven science with eyes wide open for societal relevance.

I thank you all for your attention and will finish with a few last words in Dutch: Ik wil mijn moeder en wijlen mijn vader van harte bedanken voor de onvoorwaardelijke steun die zij mij gegeven hebben tijdens mijn studie biologie en daarna. Ik dank mijn echtgenote José, mijn steun en toeverlaat in voor- en tegenspoed. Zij heeft zich laten inspireren door de kleur- en vormenrijkdom van insecten en in haar fijnzinnige kunstwerken tot uitdrukking gebracht. Mijn zoons Camiel en Martijn hebben zich ontwikkeld tot onderzoekers van de natuur en van de menselijke cognitie. Ik hoop dat jullie mij beiden nog lang van jullie ervaringen en inzichten deelgenoot blijven maken.

Ik heb gezegd.

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